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ARTIFICIAL RADIOACTIVE ANTIGENS

III. S^{35}-SULFANIL-AZO-SHEEP RED CELL STROMATA; PREPARATION AND GROSS DISTRIBUTION IN NORMAL RABBITS AND MICE

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In connection with studies on the dynamics of hemolysin formation in rabbits,[1] information on the fate of the sheep red cell antigen was desired, especially to ascertain whether differences in antibody response accompanying splenectomy, X irradiation, and intensive immunization leading to a refractory state were reflected by differences in the fate of the antigen.[1–4] The antibody response to the injection of particulate antigen apparently differs in at least some respects from that to soluble antigen.[5] Recently, the secondary response to I^{131} bovine γ globulin has been found to be resistant to X rays,[6] while that to sheep red cells is at least as sensitive as the primary response.[4] Therefore, it would also be of interest to know whether there are differences in the fate of a given hapten coupled to a particulate as compared to a soluble carrier. In order to investigate these questions, previous work on the disposition of S^{35}-sulfanil-azo-bovine-γ-globulin has been extended to S^{35}-sulfanil-azo-sheep red cell stromata.† The use of labeled stromata rather than intact cells avoids the presence of labeled soluble hemoglobin and also makes it possible to couple much more SA to the antigen then can be done with cells without causing hemolysis. The hemolysinogenic antigen of the red cell is apparently entirely in the stroma.[7]

In this paper preparation of the SA-stromata antigen and some control experiments on the fate of S^{35}A-protein in vivo are described. The hemolysin response to SA-stromata in rabbits is compared to that with intact red cells, and the gross disposition of S^{35}A-stromata in normal rabbits and mice is compared with that of S^{35}AgG in mice reported previously.[8] In a following paper[9]

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The author wishes to express appreciation to Dr. L. G. Taliaferro, who titrated the rabbit serums, and to Miss Marian S. Hill for technical assistance. Present address: Department of Microbiology, Indiana University Medical Center, Indianapolis, Indiana.

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† The following abbreviations will be used: SA, sulfanilic acid or sulfanilazo-; gG, bovine serum γ globulin; tyr, L-tyrosine; gly-tyr, glycyl-L-tyrosine; TCA, trichloroacetic acid.

1. Taliaferro, W. H. and Taliaferro, L. G., 1950, J. Infect. Dis. **87**: 37–62.
2. Taliaferro, W. H. 1951, J. Infect. Dis. **89**: 143–168.
3. Taliaferro, W. H. 1952, J. Infect. Dis. **90**: 205–232.
4. Taliaferro, W. H. and Janssen, E. F. 1952, J. Infect. Dis. **91**: 105–124.
5. Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies, ed. 2, Monograph of the Hall Institute, Melbourne, Macmillan and Co., Chap. III.
6. Dixon, F. J., Talmage, D. W. and Maurer, P. H. 1952, J. Immunol. **68**: 693–700.
7. Hadjopoulos, L. G. 1921, Arch. Int. Med. **27**: 441–448.
8. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 117–129.
9. Ingraham, J. S. 1955, J. Infect. Dis. **96**: 118–128.

the hemolysin response and gross distributions in splenectomized, intensively immunized and X-irradiated rabbits are compared to those in normal rabbits.

METHODS AND MATERIALS

Animals.—Rabbits were from Sharp at Wheaton, Illinois, and were in good condition. They were fed Dixie rabbit pellets from Dixie Mills Company, East St. Louis, Illinois, and weighed 1.7 to 2.6 kg at the time of injection. Mice were Swiss albino (Webster) from Markham Laboratories or Carworth Farms CF 1 where indicated. They were fed pelleted dog food from the Dixie Mills Company and weighed 20 to 30 g at the time of injection. Mice were autopsied as described previously.[10] Rabbits were bled to death from the heart. All injections were intravenous via the ear vein in rabbits or the tail vein in mice unless described otherwise. Calibrated syringes having a standard error of less than 4% for the volumes injected were used for all radioactive injections.

Other materials.—Saline was 0.005 *M* sodium phosphate (pH 7.4) in 0.145 *M* sodium chloride. Preparation of S^{35}-labeled sulfanilic acid has been described.[11] SA-tyr, SA-gly-tyr, and SA-histidine were prepared by adding diazotized SA to two molecular equivalents of the amino acid or peptide in the cold and then adding Na_2CO_3 to obtain pH 8 to 9. After 20 minutes in the cold, the alkaline solutions were warmed to room temperature for 30 minutes to eliminate any remaining diazo-SA and then diluted with saline and neutralized with HCl. L-tyrosine and L-histidine dihydrochloride were from Nutritional Biochemicals, Inc., Cleveland, Ohio, and glycyl L-tyrosine was from Hoffman La Roche, Inc., Nutley, New Jersey. Intact sheep and rabbit cells were labeled with radioactive phosphate as described by Wasserman et al[12] for human cells but without using aseptic precautions.

Hemolysin titrations.—Hemolytic activity of rabbit serums in 50% units per ml was determined as described by W. H. and L. G. Taliaferro.[1]

Individual mice were bled from the tail directly into a 0.2 ml serological pipette. Five hundredths ml of blood thus obtained was quickly diluted with 2.45 ml of veronal buffered saline, pH 7.4, and the serum was assumed to be diluted 1:100. The diluted blood was then allowed to clot and the serum was titrated as was rabbit serum but in 2.5 ml total volume and with proportionately three times as much complement. The titer of guinea pig complement with mouse hemolysin was found to be only about one-third of that with rabbit hemolysin. For bleedings expected to have a low titer, the serum was diluted only 1:40, titrations were made in a volume of 1 ml, and the degree of hemolysis was estimated visually.

Counting stromata.—The concentrations of stromata suspensions in percent indicate the same number of stromata per ml as in corresponding concentrations of red cells used by W. H. and L. G. Taliaferro.[1] Thus a 10% suspension contained 1.6×10^9 stromata per ml. The suspensions were counted by means of the usual red cell counting technique with the following modification. The stromata were stained without undue clumping by making dilutions with a solution prepared from 0.5 ml of 5% crystal violet in 95% ethanol diluted with 100 ml of a modified Gower's solution consisting of 4.2 g sodium sulfate, 2.8 ml glacial acetic acid and 100 ml saline. The diluted suspension was allowed to settle on the counting chamber for 3 to 4 hours in a desiccator containing wet cotton.

Samples for radioassay.—Radioactivity in the tissues was determined by counting dried homogenates on copper discs as described previously.[8] Blood samples of 0.2 ml from rabbits were drawn from the vein of the uninjected ear directly into 0.2 ml serological pipettes coated with a thin film of potassium oxalate. The blood was diluted with 1.8 ml of a solution of glue[8] containing around 20 mg/ml and 1 ml of this was plated and counted as for the tissues. Samples of zinc sulfide were mixed with a solution containing 1 mg of a suspension stabilizing agent as described previously[8] and dried under an infra red lamp without blower. In general, sufficient counts were observed to give a standard error of less than 5% for the sample minus background. For some samples of very low activity, the counting error may have been 10 to 20%.

Trichloroacetic acid precipitates of tissue homogenates.—To determine the amount of radioactivity precipitated by TCA, 5 or 10 ml of a 30% tissue homogenate in cold water were diluted to 25 ml and precipitated at once by the addition of 5 ml of 42% TCA. After several hours at room temperature and usually overnight in the refrigerator, the precipitate was washed twice with 7% TCA, twice with acetone, and twice with

10. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 109–116.
11. Ingraham, J. S. 1952, J.A.C.S. **74**: 2433.
12. Wasserman, L. R. Yoh, T. F. and Rashkoff, I. A. 1951, Lab. & Clin. Med. **37**: 342–352.

95% ethanol. A stirrer made from 12 gauge copper wire having one end flattened and twisted was used with a hand grinding tool mounted on a ring stand and controlled by a voltage regulator to resuspend and disperse the precipitate thoroughly. The washed precipitate was resuspended in water, dispersed by the addition of 0.02 ml of 50% diethyl amine per ml of final volume, made to a final volume to give about 1 ml per 0.1 ml of packed washed precipitate, and an aliquot plated to count as for the tissue homogenates. The above is a modification of the method described by Crampton and Haurowitz.[13]

Preparation of sulfanil-azo-sheep stromata.—It has been reported that simple distilled water hemolysis of human cells leaves a remainder toxic to rabbits.[14] Antiserum hemolysis of red cells yielded stromata which would not keep longer than 12 hours in the refrigerator.[15] Good yields of stromata prepared in the following way have shown no toxic effects in rabbits even on repeated injection and retain their antigenic potency for at least 3 or 4 months in the refrigerator. Clean but not strictly aseptic technique was used and the final suspensions were preserved with 0.01% merthiolate. Fresh sheep blood (not more than 2 days old) in Alsever's solution was centrifuged, and the cells were washed twice with 8 to 10 volumes of saline. Fifty ml of packed cells (10 minutes at 600 g maximum) were suspended in an equal volume of saline in a 250 ml cylinder with about 0.01 ml of capryl alcohol and were treated with cotton filtered illuminating gas by bubbling it through for 20 minutes as fast as possible without foaming over.[14] The cells, after centrifugation, were suspended to a total volume of 350 ml in half strength saline at room temperature for 30 minutes. From here on all manipulations were at 0 to 5 C. The hemolyzed suspension was centrifuged in 50 ml plastic tubes for 30 minutes at 10,000 r.p.m. (11,000 g maximum) in a Sorvall SS-1 angle head refrigerated centrifuge. The stromata were resuspended gently in the same volume of half strength saline, centrifuged for 15 minutes at 8000 r.p.m. (7000 g) and then resuspended in full strength saline for overnight in the refrigerator. The next day they were centrifuged, washed with half strength saline, resuspended by gentle swirling and transferred to fresh tubes, leaving behind a small dense pellet and some stromata still containing hemoglobin. They were then washed alternately with full strength and half strength saline until completely free of any visible pink color. Two cycles were usually sufficient. The stromata, packed at 8000 r.p.m. for 15 minutes, usually yielded 30 to 35 ml with a count of 1.8 to 2×10^{10} per ml. This number is comparable to that obtained with intact cells packed for 10 minutes at 600 g. Recently, Mitchison[16] has reported that human red cell ghosts pack to about the same volume as intact cells even when centrifuged at 116,000 g. The sedimented stromata formed a firmly packed lower layer with twice as many stromata per ml as in the loose upper layer which could be poured off, but both layers were alike with respect to microscopic appearance and behavior on resuspension and recentrifugation. The two layers were always suspended together for use in the present work.

Diazo solution for labeling the stromata was prepared from 0.64 mg of radioactive sulfanilic acid, 0.3 ml of 0.2 M hydrochloric acid, and 0.18 ml of 0.05 *M* sodium nitrite. To this, after 10 minutes at room temperature, was added 0.2 ml of 0.1 *M* urea or ammonium sulfamate, and the mixture was left at room temperature with occasional shaking for 10 minutes and then chilled to 0 to 3 C. Just before mixing with the stromata, 0.6 ml of 0.5 *M* sodium phosphate buffer pH 7.65 and 0.18 ml of 0.2 *M* sodium carbonate were added, and the solution was transferred dropwise with stirring during 2 to 3 minutes to 8 ml of 50% stromata suspension. Coupling was allowed to proceed overnight in the refrigerator although 50% took place within the first hour. Various preparations coupled 25 to 50% of the $S^{35}A$ taken and had 10 to 20 million SA molecules per stroma. The coupled stromata were washed with saline by centrifugation as described above until at least 98% of the remaining radioactivity was sedimentable. This usually required 2 or 3 washes of 35 ml each. Although 98% of the S^{35} was sedimented with the stromata in the centrifuge, only about 85% was precipitated by 7% TCA in homogenates of stromata with rabbit or mouse liver or from suspensions in rabbit serum.

Total nitrogen was determined by the modification of Groman[17] and solids by drying an aliquot of the suspension after dialysis against distilled water. Two stromata preparations averaged 8.1 mg total solids with 9.5% N_2 per 2×10^{10}

13. Crampton, C. F. and Haurowitz, F. 1950, Science, **112**: 299–302.
14. Vedder, E. 1919, J. Immunol. **4**: 141–146.
15. Sawyer, H. P. and Bourke, A. R. 1946, J. Lab. & Clin. Med. **31**: 714–716.
16. Mitchison, J. M. 1953, Exper. Cell. Res. **4**: 487–489.
17. Groman, N. B. 1951, J. Infect. Dis. **88**: 126–150.

stromata and one SA-stromata preparation similarly had 5.9 mg with 8.7% N_2. The coupled stromata packed to a smaller volume per stroma in the centrifuge than did the uncoupled preparation. Using data for the percent total solids, volume, and density of sheep cells and the percent of the cell mass in the stromata given by Ponder,[18] the total solids of sheep stromata were calculated to be 4.3 to 7.0 mg per 2×10^{10} stromata.

Stability of the S^{35}A-stromata.—When freshly washed S^{35}A-stromata were stored in saline at 0 to 3 C, 95 to 98% of the S^{35} sedimented with the stromata after 24 hours and 75% after 30 days.

The stability of 37 C in saline and in fresh rabbit blood containing 0.02 to 0.03 mg per ml of heparin was determined for two preparations in which initially over 97% of the S^{35} was sedimentable. After 1 minute, 94% sedimented, at 30 minutes 80 to 85%, and at 1 hour, 83%. Since most of the stromata are removed from the blood in vivo in much less than an hour, S^{35} was considered a reliable tracer for the intact particulate antigen with respect to blood clearance and initial tissue deposition. SA-stromata were an effective hemolysinogen after 120 to 150 days storage, as indicated by data reported below under *Antibody response.*

EXPERIMENTAL RESULTS

Fate of S^{35} from S^{35}A-stromata in vivo.—Since radioactive determinations trace directly only the S^{35}, the molecular state of the injected S^{35} in the tissues is important. It has already been found that free SA and $SO_4^=$ are rapidly eliminated from mice and could not account for the gross tissue concentration of S^{35} from injected S^{35}A-protein,[10] However, it seems possible that SA bound to protein and thus retained in the tissues might be subject to metabolic alterations which could not occur with free SA. Therefore, for the experiments on the S^{35} precipitable with TCA, it was necessary to know whether sulfur from the SA hapten might be incorporated into tissue protein and whether low molecular weight SA-containing fragments of the antigen might be bound to TCA-precipitable structures in the tissue.

To determine the amount of S^{35} of the antigen which might be incorporated into the sulfur of the tissue protein, a solution was prepared containing 0.3 mg/ml of S^{35}A-stromata and 1 mg/ml of S^{35}AgG with 4 μc total S^{35} per ml, about 80% of which was in the stromata. It was injected on three successive days intravenously and intraperitoneally into mice (0.4 ml/day) and intravenously into a rabbit (3 ml/day). One group of 3 mice and the rabbit were autopsied at 2 days, and the remaining 3 mice at 6 days after the last injection. Samples of the livers were taken to determine total radioactivity. The remainder of the mouse livers and 5 g of the rabbit liver were hydrolyzed on the steam bath with NaOH in the presence of zinc granules. Sulfide liberated with HCl was swept out and passed through 0.3 *M* HCl with a stream of nitrogen and absorbed in 0.2 *M* zinc acetate slightly acid with acetic. The ZnS was washed twice with water, decomposed with HCl, reabsorbed and washed as before and then counted. For a control, radioactive sulfate solution was injected into 2 groups of 3 mice (12 μc/mouse) and put in their drinking water (1 μc/ml) for 3 days. Both groups were autopsied 2 days after the last injection and sulfide recovered as above. For the in vitro controls, a mixture of the antigen solution or radioactive sulfate solution with normal mouse liver homogenate was hydrolyzed and sulfide recovered. The total amount of S^{35} in each hydrolysate was calculated from the counts of the tissue homogenates and the percent of this S^{35} per mg of ZnS is reported in table 1. The percent of the total S^{35} derived from tissue proteins may then be calculated if it is assumed that all sulfur in the liver is protein,

18. Ponder, E. 1948, Hemolysis and Related Phenomena, New York, Grune and Stratton.

TABLE 1.—*Radioactivity in tissue protein after injection of S^{35}A-protein or $S^{35}O_4^{=}$.*

	S^{35} A-protein				$S^{35}O_4^{=}$	
	Mice		Rabbit	Control	In vivo	Control
	2 days	6 days	2 days			
Dry weight of liver hydrolyzed in g	1.70	1.68	1.44	1.57	1.7	1.8
Percent of total S^{35} of the hydrolysate per mg ZnS*	0.022	0.029	0.030	0.010	0.62	0.0017
Percent of total S^{35} of the hydrolysate derived from sulfur of tissue protein†	0.55‡	0.89‡	0.40‡		30§	

* All samples except the rabbit and the $SO_4^{=}$ control gave at least 10 times the background count.
† Mouse liver gives an average of 66 mg of $BaSO_4$ per g dry weight by sodium peroxide fusion. For the present purpose, this was assumed to be derived entirely from protein and to be all of the same specific activity as the liberated sulfide sulfur. Some justification for the latter assumption is found in the data of Tarver and Morse[19] and Tarver and Reinhardt,[20] who reported rapid equilibration of methionine sulfur with cystine sulfur after feeding radioactive methionine to rats and dogs.
‡ Minus S^{35} in sulfide from control.
§ In a duplicate experiment 33% was found. Free $S^{35}O_4^{=}$ determined by precipitation with BA^{++} after liberation of sulfide was equivalent to 58% of the total S^{35}.

that it is of the same specific activity as the liberated sulfide, and that it is equivalent to 27 mg of ZnS per gram dry weight of liver as found from unreported determinations of the total sulfur of mouse liver. The data of table 1 indicate that about 30% of the S^{35} from sulfate remaining in the tissues for 2 days was in the sulfur of the tissue proteins and the remainder could be accounted for as free sulfate. In contrast to this, less than 1% of the S^{35} from the antigen mixture remaining after 2 or 6 days was in the tissue protein.

To ascertain whether low molecular weight antigen fragments might be taken up and bound in the tissues to give TCA-precipitable radio-activity, solutions of S^{35}A, S^{35}A-tyr, S^{35}A-gly-tyr, S^{35}A-histidine and S^{35}AgG containing the equivalent of 0.05 mg of SA per ml and a suspension of S^{35}A-stromata with 0.0006 mg SA per ml were prepared. Groups of mice were injected intravenously with 0.2 ml of one of these solutions and individual mice from each group were autopsied at intervals from 0.5 to 96 hours after injection as indicated in figure 1. All of these substances except S^{35}A were concentrated in the liver. However, the complete antigens were eliminated much more slowly than the low molecular weight compounds so that by 6 hours the concentration of S^{35} from S^{35}A-tyr and S^{35}A-gly-tyr was only

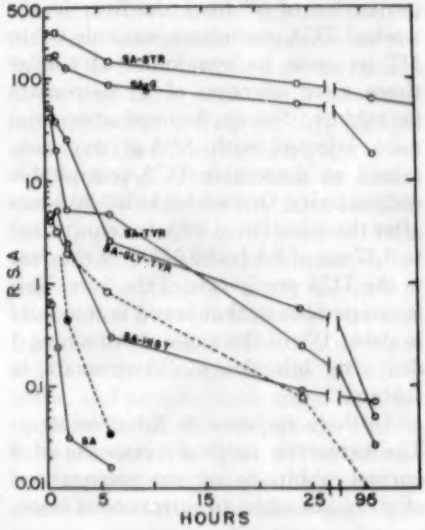

FIG. 1.—Low molecular weight sulfanilic acid conjugates in mouse liver in vivo. The relative specific activity (RSA) is the percent of the total dose per gram dry weight. Blood is assumed to be 20% solids. Broken line with open circles is the blood level from S^{35} A-tyr and S^{35}A-gly-tyr. S^{35}A-histidine blood level was somewhat lower than this up to 6 hours and about the same thereafter. Blood level from S^{35}AgG was also similar to this. Broken line with solid circles is blood level from S^{35}A. SA-str on the figure refers to S^{35}A-stromata.

19. Tarver, H. and Morse, L. M. 1948, J. Biol. Chem. **173**: 53–61.
20. Tarver, H. and Reinhardt, W. O. 1941, J. Biol. Chem. **167**: 395–400.

about 3% of that from intact antigen. The spleens had 1/5 to 1/10 of the concentration of low molecular weight compounds found in the liver. The kidney had about the same concentration as the liver, but SA-histidine, which was most rapidly eliminated from the liver, was most highly concentrated in the kidney.

To determine how much of the radioactivity from the S^{35}A-amino acid compounds retained in the tissues might be bound to TCA-precipitable material, mice were injected as above with S^{35}A-tyr or SA^{35}A-gly-tyr and autopsied as shown in table 2. The livers were homogenized in cold water and an aliquot was precipitated at once with TCA. The proportion of S^{35} from the liver in the washed TCA precipitate was only 0.2 to 1% as great as was found at similar times after injection of S^{35}A-stromata in rabbits. Spleen homogenates from mice injected with S^{35}A-gly-tyr contained no detectable TCA-precipitable radioactivity. One rabbit killed 20 hours after the injection of S^{35}A-tyr equivalent to 0.17 mg of SA had 0.046% of the dose in the TCA precipitate of the liver. This is comparable to that found in mice and is about 1% of the amount remaining 1 day after injection of SA-stromata in rabbits.

Antibody response to SA-stromata.— The hemolytic antibody response of 9 normal rabbits to various preparations of stromata after an intravenous injection of 1 ml/kg of a 10% suspension may be compared with the response found by W. H. and L. G. Taliaferro[1] for 20 normal, intact rabbits injected with a similar dose of sheep cells. At this dosage level, the response to stromata cannot be distinguished from that to intact red cells. The average peak titer of 9 rabbits given freshly prepared stromata or SA-stromata or 3-week-old SA-stromata stored in the refrigerator was 2300 (800–5600) units per ml occurring 8.4 (7 to 10) days after injection, while that of 20 with intact cells was 2830 (400 to 6000) units per ml occurring 7.6 (5 to 12) days after injection. Furthermore, the hemolysinogenic activity of SA-stromata appears to be stable for at least 3 weeks and is well within the normal range after 5 months at refrigerator temperature. Thus, three of the above rabbits were injected with SA-stromata stored for 3 weeks in saline in the refrigerator and had a peak titer of 3200 (840 to 5600) units per ml 9 days after injection, while four other rabbits injected with stromata stored for 4 to 5 months had a peak titer of 1790 (790 to 3000) units per ml 10 (8 to 13) days after injection. A fifth rabbit never attained a titer above 100 units, but such anergic rabbits have also been observed when injected with intact cells (see W. H. and L. G. Taliaferro, page 50[1]).

TABLE 2.—*TCA-precipitable S^{35} from SA-gly-tyr or SA-Tyr in mouse liver.**

	Time after injection	Percent of dose in liver	Percent of dose in TCA precipitate
SA-gly-tyr	5 min	46	0.10 ± 0.02†
	1 hr	16	0.05 ± 0.02
	6 hr	1.4	0.02 ± 0.01
SA-Tyr	6 hr		0.05 ± 0.006
	24 hr		0.03 ± 0.005

* Spleens from the mice given S^{35} A-gly-tyr had no detectable radioactivity. The liver from one rabbit killed 20 hours after injection of SA-tyr equivalent to 0.17 mg SA contained 0.046% of the dose.
† Standard error of counting.

To investigate the hemolysin response of mice to SA-stromata, two groups of Carworth farms CF 1 female mice (approximately 25 g) were injected with 0.2 ml of a 1% suspension. The groups were bled on alternate days and the resulting hemolysin titrations are plotted in figure 2. The hemolysin response was similar to that in rabbits in that it consisted of a short induction period followed by a rapid rise to peak titer and an abrupt decline. The decline indicated a maximum half-life of 1.6 days for the

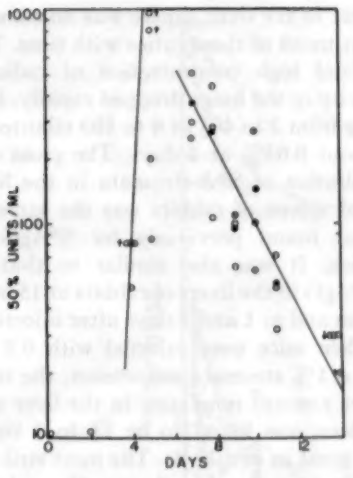

FIG. 2.—Hemolysin response of mice to a single injection of 0.2 ml of 1% SA-stromata. The arrows indicate that the actual titer was either higher or lower than could be estimated from the range of dilutions used. The solid circles are averages used for least squares calculation of the rate of decline.

hemolytic antibody. Dixon et al[21] found a half-life of 1.9 days in the mouse for the decay of homologous globulin labeled with I^{131}.

P^{32}-labeled intact sheep cells in rabbits.—An attempt was made to compare the fate of intact sheep cells with that of SA-stromata using cells labeled with P^{32}. Rabbit cells labeled in vitro and reinjected into the same rabbit remained in the circulation at the same concentration for at least 2 hours, and 99% of the P^{32} was sedimentable with the cells 1 hour after injection. These observations are consistent with the results reported by Hevesy and Zerahan.[22] However, the P^{32} of labeled sheep cells was rapidly lost into the plasma in rabbits. Two rabbits were injected with 1 ml/kg of 10% sheep cells freshly drawn and labeled by the same method used for the rabbit cells. Three samples of blood drawn from each of these rabbits at 2.5, 5 to 9, and 10 minutes after injection had only 22 to 33% of the P^{32} sedimentable with the cells. The remaining P^{32} was not bound to stromata since it did not sediment in 20 minutes at 7000 g. The distribution of P^{32} in the tissues 10 and 150 minutes after injection was similar to that found after intravenous injection of $P^{32}O^{=}_{4}$.[23,24] The liver, which accounts for 73 to 90% of the radioactivity from SA-stromata up to 100 minutes after injection, contained only 11 to 12% of the dose. The specific activity of the kidney and lung was 4 to 5 times that of the liver after 10 minutes and the intestine was almost as active as the liver. The P^{32} in the blood of two rabbits at various times after the intravenous injection of 1 ml/kg of 10% labeled sheep cells is shown in figure 3.

The fate of S^{35}A-stromata in rabbits and mice.—Radioactivity from sulfanilazo-stromata was rapidly removed from the circulation and concentrated in the tissues in rabbits. To study the rate of elimination from the blood, rabbits were injected in the ear vein with 1 ml/kg of a 1% or 10% suspension of S^{35}A-stromata, and samples were taken from the opposite ear at short intervals. The results are shown in figure 3. The increase in concentration between 10 and 100 minutes shown in the small scale plot may be the result of rapid breakdown of the stromata in the tissues and excretion of the radioactivity. Less than 5% of the radioactivity remaining in the blood longer than 10 minutes was sedimentable at speeds sufficient to remove

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23. Hevesy, G. 1948, Radioactive Indicators, New York, Interscience Publishers, Inc.
24. Hevesy, G. 1940, Danske Videnskabernes Selskab Biologiske Meddeleser 15 (no. 5): 17, Copenhagen, E. Munksgaard.

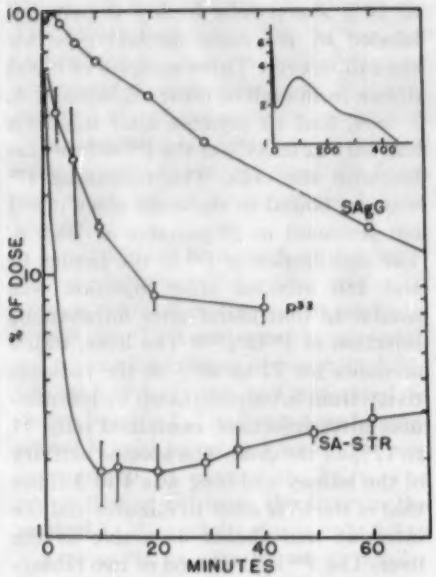

FIG. 3.—Radioactive antigens in the circulation of rabbits. Curve SAgG is data for a single rabbit given 8 mg of S^{35}AgG/kg. Curve P^{32} is an average of two rabbits given sheep cells labeled with $P^{32}O_4^{=}$. Curve SA-str is an average of three rabbits given SA-stromata. Vertical bars indicate the range. A blood volume of 70 mg/kg body weight was assumed in all calculations.[25] Insert at upper right shows data for SA-stromata up to 7 hours after injection.

stromata. For comparison, the blood level of S^{35} after injection of 8 mg/kg of S^{35}AgG into one rabbit is also plotted in figure 3.

The gross radioactivity of the liver, spleen, kidney and lungs at various times after injecting rabbits with 1 ml/kg of 1% or 10% S^{35}A-stromata suspension is shown in figure 4. The liver contained 73 to 87% of the radioactivity between 6 and 100 minutes after injection. Although the liver contained 43 (16 to 70) times as much of the activity as the spleen, the concentration in the spleen was 2.6 (1.4 to 5.6) times that in the liver. There was no consistent trend of these ratios with time. The initial high concentration of radioactivity in the lungs dropped rapidly, falling from 2 to 4% at 6 to 100 minutes to about 0.03% at 2 days. The gross distribution of S^{35}A-stromata in the liver and spleen of rabbits was the same as that found previously for S^{35}AgG in mice. It was also similar to that of S^{35}AgG in the livers of rabbits at 15 minutes and at 1 and 7 days after injection. When mice were injected with 0.2 ml of a 1% stromata suspension, the relative amount remaining in the liver and spleen was found to be 1½ to 2 times as great as in rabbits. The most striking difference was that the specific activity of the liver in mice was 4 to 6 times that of the spleen during the first 6 days, while in rabbits the specific activity in the spleen was 2 to 3 times the liver. This relation is apparently characteristic of the distribution of antigens in rabbits as compared to mice. It occurs with both soluble and particulate antigens and has been found by others.[26] It may be connected with the fact that the spleen is relatively 10 to 20 times as large in the mouse as in the rabbit. Thus, in both animals, the spleen takes up about the same percent of the total antigen.

The bone marrow, not shown in the figure, contained over 1% of the dose per gram dry weight at 10 minutes, 0.1% at 1 day and 0.04% at 7 days. The appendix and heart each contained less than 0.1% of the dose by 1½ hours after injection.

The fraction of the total activity precipitated by 7% TCA was the same in the liver and spleen—40 to 70% at 6 to 8 minutes, 25% at 1 day, and 45% at 7 days. In the kidney 40 to 50% was pre-

25. Aikawa, J. K. 1950, Am. J. Physiol. **162**: 695–702.

26. Libby, R. L. and Madison, C. R. 1947, J. Immunol. **55**: 15–26.

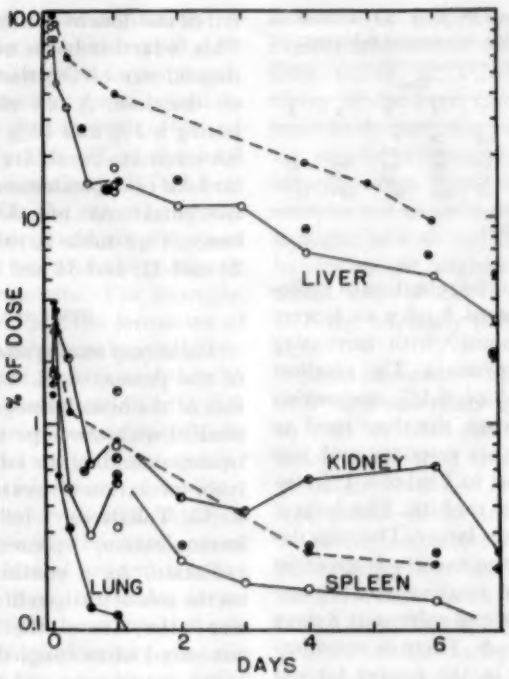

FIG. 4.—Radioactive antigens in rabbits and mice. The labeled solid lines are for tissues from rabbits injected with SA-stromata. The upper dashed line is mouse liver after injection of SA-stromata; the lower, mouse spleen. Upper circles X points are for livers and the lower for spleens of mice given SAgG as described in reference 8. The two upper solid points are for livers of rabbits given SAgG. Points for mice represent averages of 2 to 5 animals; those for rabbits are from single animals.

cipitable at both 1 and 7 days. The individual data may be found in table 2 of the following paper.[9]

Additional data on some rabbits are given in table 3.

In order to ascertain the effect of the

TABLE 3.—*Data on rabbits.*

Rabbit no.	63	30	24	22	38	34	59	36	37	23
Time killed										
Hours	0.10	0.17	1.5	1.7	6.0					
Days						1	1	4	7	12
Percent concentration of stromata infected	1	10	1	10	10	10	1	10	2	10
Weight in kg	2.2	1.7	2.0	2.4	1.8	1.9	2.2	2.1	2.0	2.9
Dry weight of:										
Liver	36	24	15	22	29	25	20	18	20	25
Spleen	0.33	0.24	0.17	0.35	0.15	0.12	0.30	0.22	0.19	0.40
Kidney	4.0	2.6	3.5	3.7	3.5	2.6	3.7	2.8	3.0	4.2
R.S.A.*										
Liver	2.41	3.02	4.94	3.96	1.18	0.59	0.91	0.37	0.16	0.08
Spleen	5.96	7.37	9.3	5.70	3.27	2.70	2.66	0.72	0.32	0.25
Kidney	0.30	0.60	0.62	1.03	0.61	0.20	0.39	0.19	0.08	0.12
Lung		0.72	1.28	2.14	0.16	0.06		0.03		0.01
Bone Marrow		1.19	0.48	0.41	0.13	0.14		0.07	0.04	0.02
Blood		0.15	0.11		0.08	0.01		0.01		

* R.S.A. = percent of the dose per g dry weight. Values for the liver and spleen were corrected for papain added during preparation of the samples.[9] This correction is less than 2% for all tissues except the spleen.

TABLE 4.—*Percent of S^{35} from SA-stromata in excreta of mice after intravenous injection.*

Relative dose	Day					
	½	1	2	4	6	8
N	77	88	96			
10 *N* (A)	72	81	89	94		
10 *N* (B)	69	82	90	94	97	97
100 *N*	70	80	89	95	97	99
1000 *N*	74	92	101	107	109	111

* See text for explanation.

size of the dose on the retention of radioactivity, 5 groups of 3 mice each were injected intravenously with increasing amounts of SA-stromata. The smallest dose was 0.2 ml of 0.1% suspension which was one-tenth the dose used to study the hemolysin response and was roughly equivalent to 1 ml of a 1% suspension per kg in rabbits. The largest dose was 1000 times larger. The cumulative recovery in the excreta is given in table 4, and the relative specific activity of the livers and spleens at 8 days is shown in figure 5. There is considerable uncertainty in the figures for excreta since the errors are cumulative. For all doses, 69 to 77% was excreted within 12 hours and 89% or more within 2 days. The data indicate that the rate of elimination was essentially independent of the dose over this 1000-fold range. This is further borne out by the lack of dependence of the tissue concentration on the dose. A few observations comparing a 1% and 10% concentration of SA-stromata in rabbits indicated a similar lack of dependence of relative tissue concentrations on dosage over this range. (See table 3, rabbits 63 and 30, 24 and 22, and 34 and 59.)

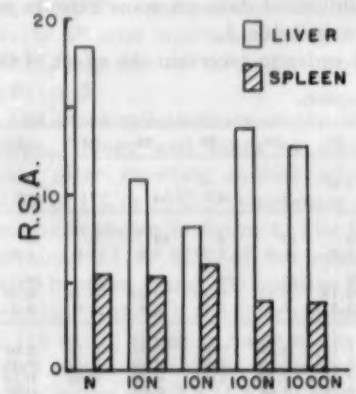

FIG. 5.—Radioactivity from various amounts of SA-stromata in mice 8 days after injection. Description in text.

DISCUSSION

Validity of tracing stromata.—The aim of the present work was twofold. The fate of the hemolysinogenic antigen was studied with the hope that it might aid in understanding the inhibition of hemolysin formation observed by W. H. and L. G. Taliaferro[1-4] following intensive immunization, splenectomy or X-irradiation. As a continuation of work on the role of antigen in antibody formation,[8] the fate of the SA hapten was compared when coupled to an insoluble particulate carrier and to a soluble one. Presumably the features common to the metabolic fate of SA on these two carriers would be most likely to have some essential relationship to antibody formation.

Inasmuch as SA is not a determining part of the antigenic structure responsible for the formation of hemolysins for normal cells, it was essential to know whether the S^{35} of sulfanilic acid (SA) is a reliable tracer for the intact stromata. Experiments on the stability of the SA to stroma bond indicate that the radioactivity of SA probably remains bound to the stromata during the brief period while they are being removed from the blood and initially deposited in the tissues and, hence, is reliable as a tracer for the initial gross deposition of antigen in the tissues. However, the stromata are probably very rapidly degraded within the host and the fate of the hemolysinogenic antigen may not be similar

to that of the SA-containing moiety. A few minutes after the intravenous injection of SA-stromata, a large proportion of the radioactivity was bound to particles smaller than stromata,[27,28] and by 24 hours only 3 to 4% of the total original dose was retained in TCA-precipitable form. On the other hand, SA itself is a strong determinant group and forms an active part of the antigenic mosaic of SA-stromata. For example, SA-proteins stimulate the formation of antibodies which behave as typical hemolysins toward red cells coupled with SA.[29] Intensive immunization with SA-stromata also gives serum specifically reactive with the sulfanil-azo group.[9] Therefore, determinations of radioactivity at longer intervals after injection are of significance for the more general problem of the fate of antigen if it can be shown that they are tracing intact sulfanilic acid. It has been reported previously that S^{35}AgG does not give rise to any appreciable amount of $S^{35}O_4^{=}$ in the urine of mice.[10] Data reported here indicate that no more than 2 to 4% of the protein-bound radioactivity from S^{35}A-protein could be accounted for by sulfur incorporated into the amino acids of the tissue proteins.* Therefore, the radioactivity is most probably a reliable measure of intact sulfanil-azo groups retained in some form in the tissue. Since S^{35}A itself or S^{35}A coupled to tyrosine, histidine or glycyl-tyrosine was very rapidly eliminated and gave at most only traces of TCA-precipitable activity in the liver, the persistent SA is most likely part of a fairly high molecular weight polypeptide or possibly an intact protein. These experiments, however, do not exclude the possibility that it might be attached to low weight molecular weight fragments of the antigen which were bound to cell proteins due to being originally part of a large molecule.

Blood clearance.—The blood clearance of S^{35}AgG was relatively slow compared to that of S^{35}-A-stromata but it was much faster than that of trace-labeled globulin.[31] Neither rapid removal nor long persistence of antigen in the blood, however, seems to have any necessary relationship to antibody formation since antigenic substances display both types of behavior.[32] Coupling with diazo compounds can markedly increase the rate of blood clearance for either serum albumin or globulin,[32,33] with no obvious corresponding effect on antigenicity. X-irradiation or splenectomy can depress antibody formation markedly with little or no effect on elimination of the antigens from the blood.[27,34] The rate of removal of SA-stromata from the blood of rabbits is similar to that found by others in early experiments with avirulent pneumococci[35] or more recently for

27. Ingraham, J. S. To be published.
28. Ingraham, J. S. 1954, Federation Proc. **13**: 236.
29. Ingraham, J. S. 1952, J. Infect. Dis. **91**: 268–75.
30. Haurowitz, F., Walter, H. and Humm, F. D. 1954, Federation Proc. **13**: 496.

* Since this paper was submitted for publication, Haurowitz et al[30] have reported that most of the radioactivity remaining in the livers of rabbits 4 weeks after injecting S^{35}AgG could be identified chromatographically as SA-tyrosine, and that less than 3% was contained in the cystine plus methionine of these livers.

31. Gitlin, D., Latta, H., Batchelor, W. N. and Janeway, C. A. 1951, J. Immunol. **66**: 451–461.
32. Dixon, F. J., Bukantz, S. C., Dammin, G. J. and Talmage, D. W. 1951, Federation Proc. 10:553.
33. Latta, H. 1951, J. Immunol. **66**: 635–652.
34. Fitch, F. W., Barker, P., Soules, K. H. and Wissler, R. W. 1953, J. Lab. & Clin. Med. **42**: 598–620.
35. Wilson, G. S. and Miles, A. A. 1946, In Topley and Wilson's Principles of Bacterology and Immunity, ed. 3, Baltimore, William and Wilkens Co. Also see Wright, H. D. 1920, J. Path. & Bact. **30**: 185.

radioactive colloidal gold suspensions.[36]

Gross distribution.—The gross tissue localization of S^{35}A-stromata is characteristic of particulate material and denatured protein. The percent of the dose retained in the liver, spleen and bone marrow of two rabbits killed at 90 and 100 minutes after intravenous injection was similar to that reported by Barrow et al[36] for colloidal gold at 1 hour. The amount in the lungs was 4 to 5 times as high as for the gold, possibly due to the larger size of the stromata; that in the kidney was 10 times as high, probably from breakdown products of the stromata; and there was a negligible amount of either gold or stromata localized in the appendix and heart.

With respect to gross tissue distribution, the fate of S^{35} A is similar whether attached to sheep stromata or to bovine serum globulin over a considerable range of dosages. The rate of excretion and the tissue retention of radioactivity from SA-stromata in mice were not influenced by the size of the dose over a 1000 fold range.

It has been suggested by W. H. and L. G. Taliaferro[1] that hemolysin formation may be proportional to antigen concentration over a limited range of concentration and that the decreased relative importance of the spleen with more intense antigenic stimulation might result from the fact that small doses of antigen were sufficient to cause maximum formation by the spleen, whereas larger doses were required for maximum stimulation of extrasplenic sites. Insofar as the concentration of antigen in an organ is a measure of relative intensity of stimulation, the present data provide some support for this suggestion in that the concentration of radioactivity in the spleen shortly after injection was about 10 times that in the bone marrow. Although the liver takes up by far the major part of the antigen injected, some recent observations suggest that it may be relatively unimportant in antibody formation.[37] If hemolysin occurs in a protein fraction not produced in the liver, the spleen and bone marrow may contain a large proportion of all the effective antigen in the body.

The data presented here provide no evidence for an abrupt disappearance of antigen from the spleen to coincide with the onset of the inactive phase in antibody production found by the Taliaferros.[1] The maximum time of persistence in mice or rabbits for native bovine globulin labeled in various ways has been reported by some workers to be from 7 to 8 days [32,38] and by others to be 100 to 200 or more days[5,39,40] The observed maximum time may depend principally upon the techniques for observation and criteria for identifying immunologically active antigen in the tissues. The assumption, apparently held by some workers, that antibody formation can proceed long after all homologous, antigenically active material has disappeared from the tissues[5,32] will be difficult to prove. Conversely, it will probably be as difficult to prove that the persistence of such material is an essential requirement for continued antibody formation. In general, there is no assurance that determinations of radioactivity from labeled antigens are actually tracing the active antigen. However, it is apparent that the fate of at least some of the SA hapten initially in-

36. Barrow, J., Tullis, J. L. and Chambers, F. W., Jr. 1951, Am. J. Physiol. **164**: 882–831.

37. Miller, L. L., Bly, C. G. and Bale, W. F. 1954, J. Exper. Med. **99**: 133–153.

38. Coons, A. H., Leduc, E. H. and Kaplan, M. H. 1951, J. Exper. Med. **93**: 173–188.

39. Crampton, C. F., Reller, H. H. and Haurowitz, F. 1953, J. Immunol. **71**: 319–324.

40. McMasters, P. D. and Kruse, H., 1951, J. Exper. Med. **94**: 323–346.

jected must be significant for antibody formation. Therefore, even though the intact stroma is probably quickly degraded within the host cell, the present observations provide evidence that the essential active determinant groups may persist and influence antibody formation for long periods of time. It remains to be determined what, if any, of the long-persisting hapten actually plays an active role in antibody formation.

SUMMARY

Sheep red cell stromata labeled by coupling with S^{35}-sulfanilic acid gave a hemolysin response in rabbits indistinguishable from that of normal sheep red cells. The hemolysin response in mice was qualitatively similar to that in rabbits. The S^{35}-sulfanilic acid was stably bound to the stromata and its radioactivity is probably an accurate tracer for the blood clearance and initial tissue deposition of the hemolysinogenic antigen of the stromata. Exchangable $P^{32}O_4^{=}$ is not a reliable tracer even for blood clearance of intact sheep cells in rabbits.

Radioactive sulfur of the antigen was not incorporated in significant amounts into the cystine of the liver or spleen in mice or in one rabbit. S^{35}A-tyr, S^{35}A-gly-tyr and S^{35}A-histidine were rapidly excreted and gave relatively little TCA-preciptable radioactivity. Therefore, in the present studies, TCA-precipitable radioactivity was probably a reliable measure of sulfanilazo hapten bound to intact protein or to high molecular weight fragments in the tissues.

SA-stromata were rapidly removed from the circulation and concentrated in the liver, spleen and kidney of rabbits. Most of the radioactivity initially taken up by the lungs and bone marrow was lost within 24 hours. The heart and appendix each took up less than 0.1% of the dose. Twenty-five to 50% of the S^{35} in the liver, spleen and kidney up to 7 days was precipitable by 7% TCA. Variation in dosage by 1000-fold in mice did not change the relative rate of excretion or the specific activity of the tissues 8 days after injection. The gross distribution of the SA hapten coupled to stromata was similar in rabbits and mice to that found previously for soluble SA-bovine serum globulin in mice.

ARTIFICIAL RADIOACTIVE ANTIGENS

IV. THE FATE OF S^{35}-SULFANIL-AZO-SHEEP STROMATA IN X-IRRADIATED, IMMUNIZED OR SPLENECTOMIZED RABBITS

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For nearly fifty years it has been known that X irradiation given prior to the injection of an antigen is usually more effective in depressing antibody formation than the same irradiation given after the injection.[1] Recently, it has been observed that subsequent to an initial intravenous injection of sheep red cells given before or up to 6 hours after heavy X irradiation, hemolysin may be formed in even larger than normal amounts although at a slower than normal rate. If the same antigen is injected 12 hours or more after the same dose of X rays, however, hemolysin formation is markedly inhibited as indicated by much lower peak titers, a longer induction period and a lower rate of formation[2] (see also Dixon et al,[3] who used bovine γ globulin). Dixon et al suggested that the antibody response may be divided into an initial radiosensitive phase limited to 12 hours or less and a subsequent radioresistant antibody production phase, and that the first phase may be associated with an adaptation of globulin synthesis to the new antigen, possibly an enzymatic adaptation such as suggested by Burnet and Fenner.[4] Taliaferro et al[2] suggested that this radioresistant phase may be concerned with localization of the antigen in the antibody-forming sites and/or early metabolism of the antigen. Kohn[5] suggested that it might be concerned with the cellular fixation of antigen.

In order to determine whether the inhibition of hemolysin formation in rabbits by X ray as well as after splenectomy and during intensive immunization observed by W. H. and L. G. Taliaferro[6–8] and by Taliaferro et al[2] might be explained in part by the effect of these procedures on removal of antigen from the blood and its initial deposition in the tissues, we studied the fate of

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1. Taliaferro, W. H. and Taliaferro, L. G. 1951, J. Immunol. **66**: 181–212.
2. Taliaferro, W. H., Taliaferro, L. G. and Janssen, E. F. 1952, J. Infect. Dis. **91**: 105–24.
3. Dixon, F. J., Talmage, D. W. and Maurer, P. H. 1952, J. Immunol. **68**: 693–700.
4. Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies, ed. 2, Monograph of the Hall Institute, Melbourne, Macmillan and Co., 142 pp.
5. Kohn, H. I. 1951, J. Immunol. **66**: 525–533.
6. Taliaferro, W. H. and Taliaferro, L. G. 1950, J. Infect. Dis. **87**: 37–62.
7. Taliaferro, W. H. 1951, J. Infect. Dis. **89**: 143–168.
8. Taliaferro, W. H. 1952, J. Infect. Dis. **90**: 205–232.

S^{35}-sulfanil-azo-sheep red cell stromata. The present paper reports results for blood clearance and gross tissue deposition. Distribution in subcellular fractions of the liver will be reported in a subsequent paper. It has already been found that SA-stromata give a hemolysin response in normal rabbits similar to fresh intact sheep red cells (called sheep cells hereafter).[10]

Recently, Fitch et al[9] reported that X irradiation had no effect on the uptake and gross tissue localization of radioactivity from I^{131}-labeled typhoid bacilli.

MATERIALS AND METHODS

Most of the materials and methods, including the preparation of the radioactive and nonradioactive sulfanil-azo-sheep red cell stromata (SA-stromata) antigen, have been described previously.[10] This antigen was used exclusively in the present study and was always injected intravenously. Hemolysin was determined in 50% units/ml.[7]

Normal and X-rayed rabbits were the same as described in part III[10] of this series. Rabbits for immunization and for splenectomy were obtained from the Harold Swift farm at La Porte, Indiana, and were snuffles-free. Rabbits were irradiated as described previously[8] and were injected with antigen 1 to 3 days after 500 or 600 r. Splenectomized rabbits were injected 6 hours after the operation to study the hemolysin response and 4 to 14 days after the operation to study antigen distribution. For the repeatedly injected rabbits, the radioactive injection was the last or next to last in figure 2. Except for the 4 normal and X-rayed pairs identified in figure 4, the normal, X-rayed, immunized and splenectomized series were done at different times and various stroma preparations were used. Protein-bound radioactivity was determined by precipitation with 7% trichloroacetic acid (TCA) as described in the preceding paper.[10]

EXPERIMENTAL RESULT

Antibody response to SA-stromata.—The effect of splenectomy on the secondary response was determined in four rabbits for which the primary response was reported previously.[10] These rabbits were splenectomized 49 to 78 days after the initial injection and were reinjected the day of the operation with 1 ml/kg of 10% SA-stromata. As may be seen in figure 1, peak titer and the day of peak titer of these four rabbits were similar to the results reported by W. H. and L. G. Taliaferro[8] for three rabbits (#106, 107 and 109) splenectomized and reinjected with 1 ml/kg of 10% sheep cells 112 days after an initial injection of sheep cells. Less hemolysin was produced in all but one rabbit. In this one, the peak after splenectomy was slightly higher than before—a result also obtained with sheep cells (cf. rabbit 140 of reference 8, which was splenectomized and reinjected with 1.25 ml of 1% cells 24 days after an initial injection).

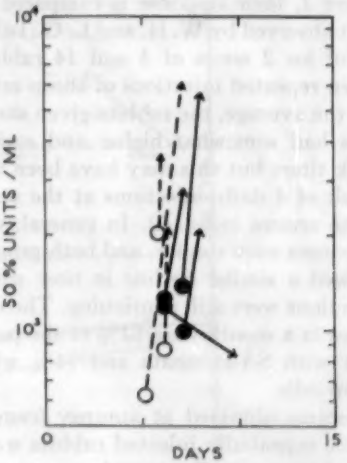

FIG. 1.—The effect of splenectomy on the hemolysin response of rabbits injected with SA-stromata or sheep cells as shown by peak titer. Solid circles indicate peak titer from splenectomized rabbits reinjected with SA-stromata, and solid lines point to peak titer for the initial injection prior to splenectomy. Open circles and dashed lines indicate similar data from splenectomized rabbits reinjected with sheep cells as reported by W. H. and L. G. Taliaferro.[8]

9. Fitch, F. W., Barker, P., Soules, K. H. and Wissler, R. W. 1953, J. Lab. & Clin. Med. **42**: 598–620.
10. Ingraham, J. S. 1955, J. Infect. Dis. **96**: 105–117.

Five intact rabbits were reinjected at the same time with SA-stromata. All responded with significantly higher and earlier peak titers than after the initial injection. The average initial peak was 1850 units at 9 (8 to 10) days, while the peak after reinjection (minus the titer at the time of reinjection) was 5690 units at 5.2 (5 to 6) days. The statistical significance for paired individual differences in peak titer was 0.03. Four of 9 rabbits gave a higher than initial titer on reinjection with sheep cells after 45 to 112 days as reported by the Taliaferros,[8] and the time to peak was reduced from 8.1 (5 to 12) to 4.2 (4 to 5) days.

To determine the hemolysin response to repeated injections of SA-stromata, 6 rabbits were injected three times a week for 30 to 35 days with 1 ml/kg of a 10% suspension of SA-stromata. In figure 2, their response is compared to that observed by W. H. and L. G. Taliaferro[8] for 2 series of 3 and 14 rabbits given repeated injections of sheep cells. On the average, the rabbits given sheep cells had somewhat higher and earlier peak titers but this may have been the result of 4 daily injections at the start (note arrows in fig. 2). In general, the responses were similar, and both groups showed a similar decline in titer while injections were still continuing. The declines in a month were 62% of the peak titer with SA-stromata and 74% with sheep cells.

Serums obtained at autopsy from 4 of the repeatedly injected rabbits were tested for antibodies homologous to the sulfanil-azo group by mixing 0.25 ml with amounts of various antigens equivalent to 0.5 μg of N_2 and then incubating 30 minutes at 37 C and overnight at 3 to 5 C. All serums gave visible precipitation with sulfanil-azo-bovine serum γ globulin but not with arsanil-azo-bovine serum γ globulin or plain bovine

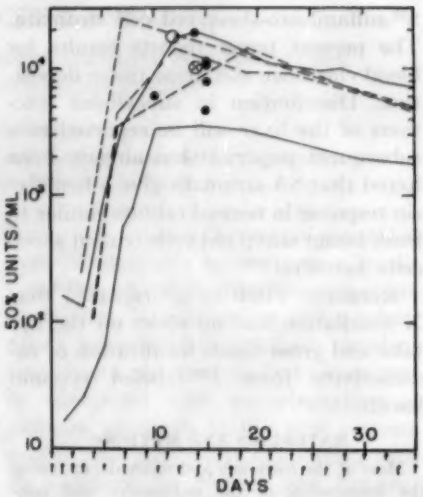

FIG. 2.—Hemolysin response in intact rabbits which received repeated injections of SA-stromata (upper row of arrows) or intact sheep cells (lower row of arrows). The two solid lines represent the range in response to SA-stromata and the solid circles represent peak titers. The circled X indicates the average of these peak titers. The dashed lines represent the response to intact cells for three rabbits with the open circle indicating an average peak titer for 14 rabbits as reported by W. H. and L. G. Taliaferro.[7] Note that repeated injections of the two antigens gave essentially similar results.

serum γ globulin. The precipitation with sulfanil-azo-globulin was inhibited by 0.1 ml of 0.01 *M* sulfanil-azo-tyrosine. The formation of antibodies homologous to hapten groups coupled to stromata has been observed previously by Landsteiner and van der Scheer.[11]

The hemolysin response in three rabbits injected with 1 ml/kg of 1% SA-stromata 1 to 3 days after 600 r was almost completely suppressed in agreement with results obtained by Taliaferro et al[2] with sheep cells.

From the foregoing studies, it may be concluded that SA-stromata act essen-

11. Landsteiner, K. and Van der Scheer, J. 1936, J. Exper. Med. **63**: 325–339.

tially similarly to sheep cells as an antigen under the following conditions: in splenectomized and intact reinjected animals, in repeatedly immunized rabbits and in rabbits that were irradiated with 600 r 1 to 3 days previously. The differences observed are probably only a reflection of the high variability frequently encountered by the Taliaferros even in similarly treated groups of rabbits.

Elimination of S^{35}A-stromata from the blood.—The rate of disappearance of 1 ml/kg of 10% S^{35}A-stromata from the blood was determined as described previously[10] in 2 rabbits* which had received 500 r of X irradiation 1 to 3 days before injection, in 2 immune rabbits and in 2 splenectomized rabbits. These procedures had little effect on blood clearance with this antigen (fig. 3). Although initial removal was somewhat faster and more nearly complete in the normal rabbits, no more than 3% remained in the blood in any of the rabbits 20 minutes after injection except in the immune group. In this latter group, the

* One X-rayed rabbit which did not show a minimum in blood concentration during the first hour was omitted from the graph to avoid obscuring what appeared to be the more typical behavior.

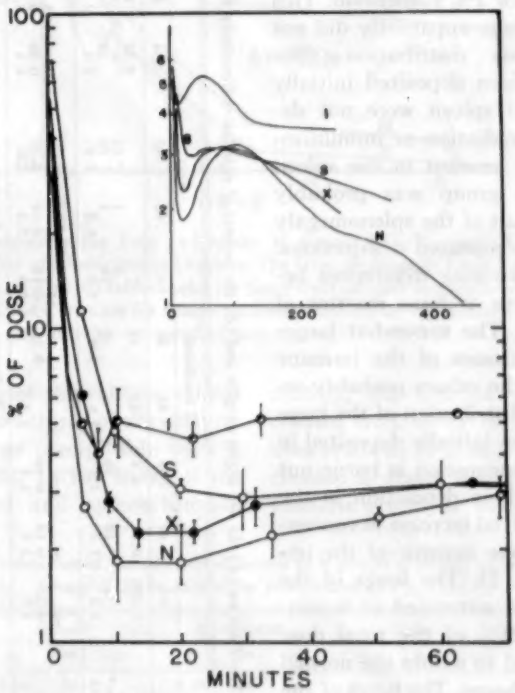

FIG. 3.—Removal of S^{35}A-stromata from the blood of normal (N), X-rayed (X), immunized (I), and splenectomized (S) rabbits. The experimental points are averages from 3 normal rabbits and from two rabbits for the other categories. The vertical bars indicate maximum range. The percent of dose in the blood was calculated on the assumption of a blood volume of 70 ml/kg. On the small scale insert the axes are the same as for the main graph except for a compressed time scale. The normal curve beyond 100 minutes is for one rabbit only. One X-rayed rabbit which did not show the initial minimum was omitted from the graph.

higher concentration may have resulted from a relocation of the abnormally high amount deposited in the lungs (see below).

Deposition of S^{35}A-stromata in the tissues.—Gross distribution of radioactivity of S^{35}A-stromata in the tissues of normal, X-rayed, immunized or splenectomized rabbits are shown in figures 4, 5 and 6, and additional data for some of the rabbits are given in table 1. Similar data for normal rabbits are given in table 5 of a preceding paper.[10] The test injection was usually 1 ml/kg of 10% S^{35}A-stromata, but in 4 of the X-rayed rabbits and in the controls it was 1 ml/kg of either a 1 or 2% suspension. This difference in dosage apparently did not affect the gross distribution. The amounts of antigen deposited initially in the liver and spleen were not decreased by X irradiation or immunization. The larger amount in the spleen of the immune group was probably principally a result of the splenomegaly accompanying repeated injections. There were no striking differences between the kidneys or bone marrow of the four groups. The somewhat larger amount in the tissues of the immune rabbits than in the others probably resulted from a redistribution of the large amount of antigen initially deposited in the lungs. This suggestion is borne out by the later peak in deposition in the spleen and the initial increase in concentration in the bone marrow of the immune group (fig. 5). The lungs of the immunized rabbit autopsied at 6 minutes contained 25% of the total dose but this decreased to within the normal range by 6 to 12 hours. The lungs of the X-rayed rabbits contained less radioactivity than those of the normal for the first few hours after injection.

The percent of the radioactivity bound to protein in homogenates of the livers, spleens and kidneys is given in

TABLE 1.—*Data on (A) the dry organ weights and (B) the percent of injected radioactivity per gram dry weight in 3 groups of rabbits.*

Rabbit no.	X irradiated									Immune					Splenectomized			
	62	49	46	83	56	47	85	42	594	595	599	597	598	96	97	98
Time killed: Hours	0.10	0.12	6.4	22	23				0.12	1.1	6.2			0.14	24	
Days						3	7	12				2.8	7			7
Percent concentration*	1	10	10	2	1	10	2	10	10	10	10	10	10	10	10	10
Weight of rabbit in kg	2.2	2.2	2.1	1.6	2.0	1.9	1.8	2.4	2.2	2.1	1.8	2.4	2.3	2.6	2.6	3.7
A. Dry weight (grams)																
Liver	20	40	17	15	15	15	15	26	32	25	25	36	22	23	24	22
Spleen	0.21	0.13	0.19		0.13	0.10	0.18	0.22	0.30	0.27	0.32	0.41	0.52			
Kidney	3.7	3.9	3.4	2.2		2.6	2.3	3.6	4.4	3.8	3.6	4.6	4.6	3.4	3.3	4.4
B. Percent of injected radioactivity per gram dry weight																
Liver	4.82	1.82	2.84	1.12	1.02	0.87	0.50	0.13	1.93	2.32	1.70	0.30	0.30	2.97	0.94	0.48
Spleen	4.48	5.53	9.26		2.69	1.17	0.57	0.18	2.88	2.77	7.70	1.54	0.90			
Kidney	0.26	0.34	0.55	0.22	0.41	0.34	0.16	0.12	0.27	0.49	0.58	0.26	0.18	0.92	0.33	0.25
Lung		0.37	0.14			0.06		0.09	15.7	10.4	1.28	0.02	0.00	4.47	0.01	
Bone marrow		0.56	0.42			0.11	0.04	0.03	0.18	0.17	0.44		0.18	0.58	0.15	0.08
Blood		0.16	0.05			0.01		0.00	0.14	0.20	0.15	0.00	0.00	0.48	0.02	0.00

* 1 ml/kg rabbit of this concentration was injected intravenously.

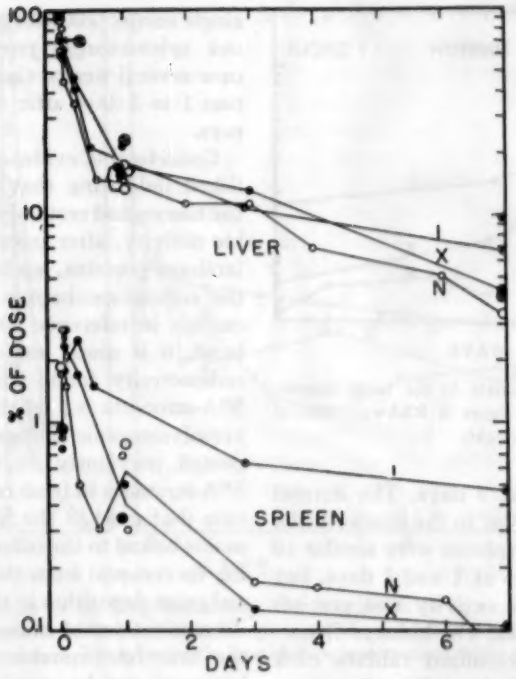

FIG. 4.—S^{35}A-stromata in the liver and spleen of normal (N), X-rayed (X) or immunized (I) rabbits and in the liver of splenectomized rabbits. The large circles at 0, 1 and 7 days indicate results for pairs of normal and X-rayed rabbits used for tissue fractionation studies to be published. The circled X points at 0, 1 and 7 days are for splenectomized rabbits. The "% of dose" refers to the percent of the total injected radioactivity recovered in each organ.

table 2 for some of the rabbits. About one-quarter to two-thirds of the activity in the tissues was precipitable by 7% trichloracetic acid. In the livers of the normal, X-rayed and splenectomized rabbits, the fraction precipitated decreased from 60 or 70% at 6 to 8 minutes to about 25% at 1 day and then increased to about 40% by 7 days. In the immune group, it was 62% at 7 min-

TABLE 2.—*Protein-bound radioactivity of tissues in 4 groups of rabbits as indicated by the percent of S^{35} in each tissue precipitated by 7% TCA.**

Rabbit no.	Normal			X irradiated			Immune			Splenectomized		
	63	**	87	62	**	85	594	595	597	96	97	98
Time killed: Hours	0.10	24		0.10	23		0.12	1.1		0.14	24	
Days			7			7			2.8			7
Liver	42	25	44	61	26	40	62†	36†	26†	69†	27	38
Spleen	38	25	47	19	23	60						
Kidney	30‡	50	46	§	28	40					40	50

* Precipitates were prepared as described in paper III[10] from aqueous homogenates, except as indicated.
† From homogenate in 0.88 *M* sucrose.
‡ 20% standard error of counting.
§ Too low to measure; less than 6% of total.
** Normal spleen was from #59; liver and kidney are an average of 86 and 59. X irradiated spleen was from #56, liver and kidney are an average of #83 and 56. Maximum deviation was 14% of the average.

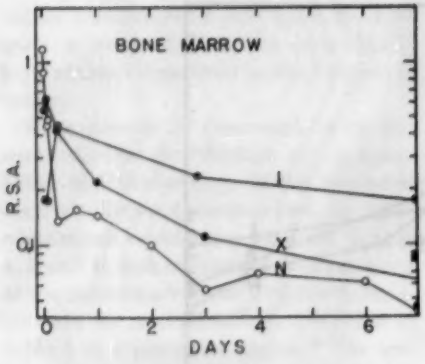

FIG. 5.—S^{35}A-stromata in the bone marrow. For description, see figure 4. RSA = per cent of dose per gram dry weight.

utes and 26% at 3 days. The normal spleens were similar to the livers. In the X-rayed group, spleens were similar to the normal group at 1 and 7 days, but only 19% of the activity was precipitated at 6 minutes. The kidneys of normal and splenectomized rabbits each had about half of the radioactivity precipitable at 1 and 7 days. The amounts of protein-bound radioactivity in the kidneys of the X-rayed animals at 6 minutes and 1 day were apparently significantly lower than normal.

DISCUSSION

The hemolysinogenic antigen of sheep red cells is substantially all retained by the stromata after hemolysis.[12] Data presented in this and a preceding paper,[10] as compared to data on fresh intact sheep red cells by the Taliaferros,[2,5–8] indicate that even by the sensitive 50% hemolytic method the hemolysin response was essentially similar with SA-stromata and sheep cells in doses of 1 ml/kg of a 10% suspension. These observations were made on rabbits given either a single intravenous injection, a single reinjection with or without previous splenectomy, repeated injections over several weeks, and an initial injection 1 to 3 days after 600 r of hard X-rays.

Considerable evidence has been published indicating that radioactivity in the tissues and certainly TCA-precipitable activity, after injection of S^{35}A-sulfanil-azo-proteins, is a reliable tracer for the sulfanil-azo-hapten group (see discussion in reference 10). On the other hand, it is much less certain that the radioactivity found after injection of S^{35}A-stromata is a reliable tracer for the hemolysinogenic antigen. Studies reported previously on the stability of S^{35}A-stromata in fresh rabbit blood indicate that most of the S^{35}A probably remains bound to the intact stromata during its removal from the blood and initial gross deposition in the tissues.[10] The determinations of radioactivity beyond the first few minutes after injection, however, can be considered a reliable indication only of the fate of the sulfanil-azo-hapten originally coupled to the particulate carrier. Similar considerations must apply to any work attempting to correlate antibody formation with the fate of antigen when the antibody is formed against structures in the antigen other than those directly traced by the radioactivity. We are at present investigating the effect of X irradiation on the formation of antibody specific for the SA-hapten group.

Data for the disappearance of the radioactivity of P^{32}-labeled sheep cells from the blood of rabbits suggest that intact cells are rapidly removed,[10] and that the initial fate of the Forssman hapten from intact cells may be the same as that from stromata. In any event, the explanations which have been proposed for the effects of X rays, splenectomy and immunization on the hemolysin response to red cells[2,5–8]

12. Hadjopoulos, L. G. 1921, Arch. Int. Med. 27: 441–448.

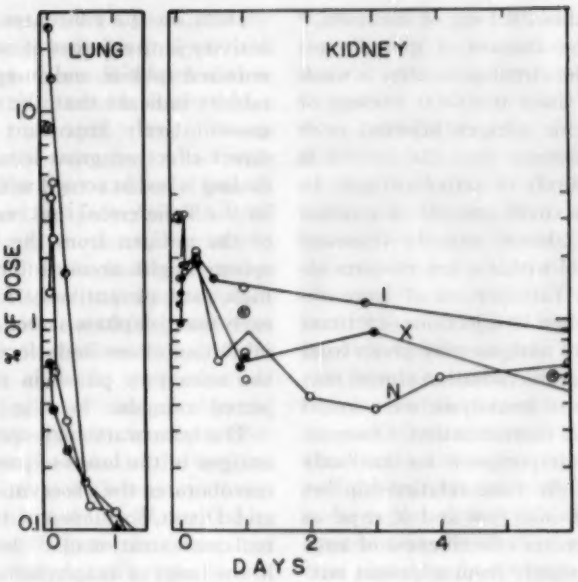

FIG. 6.—S^{35}A-stromata in the lung and kidney. For description, see figure 4. The point for the kidney of a splenectomized rabbit at the right margin is at 7 days. Each of the other groups also had an additional point at 7 days. The value for the lung of the splenectomized rabbit at 1 day was 0.03%.

should hold also for the similar effects on the response to SA-stromata under comparable conditions.

None of the procedures studied had any marked effect on removal of antigen from the blood. As discussed in a preceding paper, the rate of clearing of this antigen was essentially the same as that observed for radioactive colloidal gold by Barrow et al[13] and was similar to that of nonvirulent pneumococci observed by Wright.[14] Barrow et al also found that X irradiation had no effect on the clearing of colloidal gold from the blood. Therefore, these data do not support a suggestion of the Taliaferros[8] that the shorter induction period for the secondary response might be explained in part by a more rapid removal of antigen from the blood of the immune animals such as occurs for some soluble antigens.[15]

The radioresistance of the secondary response to bovine globulin found by Dixon et al[3] as compared to the radiosensitivity of the secondary response to red cells found by Taliaferro et al[2] may be explained in part by the rapid removal of S^{35}A-stromata from the blood compared to the persistence of I^{131}-labeled bovine globulin in the blood of X-rayed rabbits. A single injection of 30 mg/kg of the trace-labeled I^{131} bovine globulin used by Dixon et al would give about 1 mg/kg still in the circulation 7 to 8 days after injection in the absence of the immune response (from fig. 4 in reference 15). The dose of 1.25 ml of 1% red cells used by Taliaferro et al con-

13. Barrow, J., Tullis, J. L. and Chambers, F. W. 1951, Am. J. Physiol. **164**: 822–832.
14. Wright, H. D., 1927, J. Path. & Bact. **30**: 185–252.
15. Talmage, D. W., Dixon, F. J., Bukantz, S. C. and Dammin, G. J. 1951, J. Immunol. **67**: 243–255.

tained only about 0.1 mg of stromata.[10] Therefore, the amount of globulin remaining in the circulation after a week would be 10 times the total amount of hemolysinogenic antigen injected even if we could assume that the stroma is composed entirely of active antigen. In addition, this small amount of stromal antigen was almost entirely removed from the blood within a few minutes after injection. Taliaferro et al[2] have observed that when 10 injections of 8 times this amount of antigen were given from 2 to 17 days after irradiation almost normal amounts of hemolysin were found even on initial immunization. Observations on the importance for antibody formation of the time relationship between antigen injection and X rays[2] as well as the greater effectiveness of antigen liberated slowly from adjuvant mixtures compared to the same antigen given intravenously in a single dose[16] suggest that the most important stimulation to antibody formation comes from the initial uptake and early metabolism of the antigen. This type of stimulation could occur for several days or even more than a week after injection of a single dose of I^{131} globulin in X-irradiated animals[15] and may be as effective as repeated injections of cells.[2] This explanation may be supplementary rather than an alternative to one based on the difference in chemical nature between the Forssman and protein antigens and between the corresponding antibodies.*

Data on the concentration of radioactivity in the tissues of normal and immunized rabbits and in splenectomized rabbits indicate that the spleen has no quantitatively important direct or indirect effect on gross localization. This finding is not in accord with a suggestion by the Taliaferros[7] that removal of most of the antigen from the blood by the spleen might account for the spleen's high rate of antibody formation and early inactive phase as well as the spleen's inhibition of antibody formation during the refractory phase in repeatedly injected animals.

The temporary high concentration of antigen in the lungs of immune animals corroborates the observation of Warren and Dixon,[17] who found twice the normal concentration of I^{131} bovine globulin in the lungs of anaphylactic guinea pigs. The somewhat later peak of concentration in the spleen, the smaller amount in the liver at 6 minutes, and the increase in concentration in the bone marrow between 1 and 6 hours in the immune as compared to the normal group might result from a relocation of the antigen originally in the lungs. This, however, would probably not have any

16. Talmage, D. W. and Dixon, F. J. 1953, J. Infect. Dis. **93**: 176–180.

* Data in two recent papers by the Taliaferros (1954, J. Infect. Dis. **95**: 117–133, 134–141) indicate that the apparent resistance to X rays obtained by repeated injections of red cells from 2 to 17 days after irradiation is due to partial recovery from X-ray injury during the time of immunization. Although the amount of antibody formed is less than in nonirradiated animals given a similar course of injections, it is of the same order of magnitude as that of controls receiving a single injection. Since bovine globulin is taken up from the circulation continuously for several days after a single injection,[16] the response to this antigen might be even more sensitive to any slight recovery after irradiation. The fact that this response can be detected after reinjection but not after an initial injection following X irradiation may be a result of the more intense secondary response commonly obtained with soluble antigens. Sulfanil-azo-bovine globulin is rapidly removed from the circulation and some preliminary work on the formation of antibody to the sulfanil-azo group of this antigen (M. S. Hill, Master's Thesis, University of Chicago, June, 1954) indicates that the anamnestic response to this antigen is completely suppressed by 600 r of hard X rays given 24 hours before injection.

17. Warren, S. and Dixon, F. J. 1948, Am. J. Med. Sc. **216**: 136–145.

important depressant effect on antibody formation. Even if all the antigen held up in the lungs were destroyed rather than relocated, it would cause a decrease of less than half in the amount available to other sites, whereas relatively little effect on antibody formation was caused by a decrease to 1/80 of this amount in the total dose of red cells injected. The absolute amount retained in the tissues is proportional to the dose for at least a 10-fold reduction in dose as indicated previously.[10] It seems possible that the suppression of the initial uptake of antigen by the lungs in X-rayed animals might contribute to the suppression of the immune response. Anker and Green found significant anti-ovalbumin formation by both spleen and lungs in vitro compared to very little formation by the liver.[18]

From the data of table 2, it appears that X ray, splenectomy or immunization did not have any marked effect on the proportion of protein-bound radioactivity in the liver or spleen. The only discrepant value is that for the spleen of the X-rayed rabbits at 6 minutes.

A larger proportion of the radioactivity in the kidney was bound to protein in the normal than in the X-rayed rabbits up to at least 1 day after injection. This might be a result of the decrease in inhibitors for certain intracellular proteolytic enzymes following high doses of X rays as reported by Feinstein and Ballin.[19] The total amount of radioactivity was somewhat higher in the liver of the X-rayed animals than in the normal after 6 hours. However, it does not seem that differences of this magnitude would be significant for antibody formation unless they reflect larger differences to be found at a finer level of organization.

18. Anker, H. S. Personal communication.
19. Feinstein, R. N. and Ballin, J. C. 1953, Proc. Soc. Exper. Biol. & Med. **83**: 6–10, 10–14.

The similarity of blood clearance and tissue localization in X-rayed and normal rabbits is in general agreement with the results of Fitch et al.[9] In neither of these studies, however, is the active antigenic determinant homologous to the antibody in question being traced directly by the radioactive determinations. Therefore, the fate of the radioactivity may not parallel that of the active antigen for more than a brief interval after injection. The effect of timing of X irradiation with respect to antigen injection as discussed in the introduction seems to require that X rays have an effect at some level of organization on the interaction between antigen and antibody-forming sites. It is hardly to be expected that this effect would be apparent at the level of gross distribution since antibody responses may be stimulated by materials which remain in the circulation for days with no significant concentration by the tissues[20] as well as by materials which are rapidly cleared from the blood and concentrated in the reticulo-endothelial system.

SUMMARY

X rays or splenectomy prior to antigen injection caused marked inhibition of the hemolysin response to radioactive sulfanil-azo-sheep-stromata (S^{35}A-stromata) in rabbits. During the continued injection of SA-stromata three times a week, the titer reached a peak after 10 to 15 days and then declined in spite of antigen injections. These observations are in quantitative as well as qualitative agreement with similar ones for intact sheep cells.

Five-hundred r of hard X rays given to rabbits 1 to 3 days before injection did not affect the rate or extent of re-

20. Dixon, F. J., Bukantz, S. C., Dammin, G. J. and Talmage, D. W. 1951, Federation Proc. **10**: 553–557.

moval of radioactivity of S^{35}A-stromata from the circulation. The retention of radioactivity in the liver, spleen, kidney and bone marrow was also unaffected or possibly slightly increased. The proportion of the radioactivity in the liver which was precipitable by 7% trichloracetic acid was also unaffected.

In highly immune rabbits, radioactivity was at first markedly concentrated in the lungs with subsequent rapid relocation. The slight differences from the normal in blood clearance and gross radioactivity of the other tissues are believed to be the result of this first effect.

Splenectomy slightly decreased the efficiency of blood clearance initially as evidenced by a slower decrease prior to one hour, but had little or no effect on concentrations in the tissues.

Since rabbits respond to SA-stromata with the formation of antibodies specifically reactive with the SA group, determinations of radioactivity which trace this hapten group are of significance for the in vivo fate of antigenic material independently of the continued in vivo integrity of the whole SA-stromata complex.

Experiments reported previously on the stability of S^{35}A-stromata led to the conclusion that radioactivity is a reliable tracer for the intact stromata with respect to blood clearance and initial gross tissue deposition. Therefore, it is concluded here that suppression of hemolysin formation by X rays or splenectomy does not depend upon suppression of removal from the blood stream or initial gross tissue uptake of the antigen in the liver, spleen, kidney or bone marrow. The abrupt decrease in antibody formation during intensive immunization with SA-stromata is also probably independant of any effect on gross antigen distribution in these organs. The possible significance of differences in localization in the lungs is discussed. A possible explanation has been offered for the apparent resistance to X rays of the secondary response with globulin compared to that with red cells.

A SUBFRACTION FROM RABBIT LIVER MITOCHONDRIA WHICH RETAINS A HIGH CONCENTRATION OF RADIOACTIVITY FROM S^{35}-SULFANIL-AZO-PROTEINS

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Precise information about the uptake of antigens by the subcellular components of cells may lead to a better understanding of the mechanism of formation of both antibody and normal protein. Following the studies of Crampton and Haurowitz[1] on the intracellular distribution of I^{131}-iodo-ovalbumin, a variety of antigens have been found to be concentrated in the mitochondrial fraction of the livers of rabbits or mice. Gitlin et al[2] observed microscopically visible colored granules concentrated in the mitochondrial fracton from mouse livers after injecting various dye azo-proteins. However, they reported that these granules did not take the usual mitochondrial stains and they questioned whether the dye proteins were actually attached to mitochondria. The present author[3] found the concentration of radioactivity from S^{35}-sulfanil-azo bovine serum γ globulin in the mitochondrial fraction of mouse liver to be 1.6 times that of the whole homogenate one day after injection. Fields and Libby[4] found the concentration of radioactivity in mitochondria to be 4 to 5 times that in the whole liver protein of immune rabbits after injecting radioiodinated human serum albumin. They observed similar concentration ratios with P^{32}-labeled tobacco mosaic virus in normal mice. The gross visible heterogeneity of the mitochondrial fraction prepared by the usual methods of tissue fractionation has been emphasized in recent reports and the distinct biochemical and morphological characteristics of several subfractions, in particular, a pink, fluffy upper layer and a tan, dense lower layer, have been described.[5] The mitochondria from rat and mouse livers have recently been separated into 3 to 5 and 5 or 6 subfractions, respectively, by modified centrifugation techniques.[6,7]

This report is concerned with a small, compact sediment (Sed) resistant to resuspension, which forms below the dense mitochondria when homogenates of rabbit liver in 0.88 *M* sucrose are centrifuged. It may be similar to the fraction B 3 obtained by packing mouse liver mitochondria as described by Kuff and Schneider[7] and is clearly shown in tube 1 in figure 6 of a paper by Potter et al[8]

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1. Crampton, C. F. and Haurowitz, F. 1950, Science, **112**: 300–303.
2. Gitlin, D., Landing, B. H. and Whipple, A. 1951, Proc. Soc. Exper. Biol. & Med. **78**: 631–635.
3. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 117–29.
4. Fields, M. and Libby, R. L. 1952, J. Immunol. **69**: 581–586
5. Laird, A. K., Nygaard, O., Ris, H. and Barton, A. D. 1953, Exper. Cell. Res. **5**: 147–160.
6. Novikoff, A. B., Podber, E., Ryan, J. and Noe, E. 1953, J. Histochem. & Cytochem. **1**: 27–46.
7. Kuff, E. L. and Schneider, W. C. 1954, J. Biol. Chem. **206**: 677–685.
8. Potter, V. R., Rechnagel, R. O. and Hurlbert, R. B. 1951, Federation Proc. **10**: 646–653.

as a small dark disk at the bottom of the precipitate, although it is not described as a separate fraction. This sediment binds and retains an unusually high concentration of trichloracetic acid (TCA)-precipitable radioactivity from sulfanil-azo-sheep stromata (SA-stromata) and sulfanil-azo-bovine serum γ globulin (SAγG) labeled with S^{35}-sulfanilic acid.

MATERIALS AND METHODS

Rabbits were injected via the ear vein with 1 ml/kg of a 10% suspension of SA-stromata or a total of 5 to 10 mg of SAγG containing 5 to 8 million cpm, equivalent to 3 to 5 microcuries of S^{35}. The preparation of radioactive SAγG has been described[9] and details of the preparation of S^{35}A-stromata are in press.[10] At autopsy, rabbits were bled from the heart and their livers removed and chilled in saline ice within 5 to 6 minutes after the start of bleeding. The chilled livers were forced through a chromel wire gauze, and homogenates were prepared and fractionated as described by Hogeboom et al,[11] except that 140 or 210 ml of the 10% homogenate were fractionated. After washing the mitochrondrial fraction once with sucrose by resuspending and recentrifuging, the upper pink layer was removed with a pipette. Most of the heavy tan layer was then suspended by swirling and the remainder which adhered to the compact pellet (Sed) was washed off with a vigorous stream of sucrose from a pipette. A clean separation of the pink and tan fractions such as described by Laird et al[8] was not attempted. The separated Sed fraction was resuspended in sucrose and recentrifuged. Protein recipitates from the fractions were prepared as described by Haurowitz and Crampton,[12] except that the final washed TCA precipitates were resuspended in water and dispersed by the addition of diethylamine so that aliquots could be plated directly as described previously.[9] For the control experiments, a mi:ce of liver from a noninjected rabbit was mixed with S^{35}A-stromata or S^{35}AγG in sucrose and then homogenized and fractionated.

9. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 109–116.
10. Ingraham, J. S. J. Infect. Dis. In press.
11. Hogeboom, G. H., Schneider, W. C. and Pallade, G. E. 1948, J. Biol. Chem. **172**: 619–635.
12. Haurowitz, F. and Crampton, C. F. 1952, J. Immunol. **68**: 73–85.

EXPERIMENTAL RESULTS

Usually the sediment fraction was composed chiefly of a clear to milky translucent material, probably paricu-late glycogen, in the outer surface of which was embedded a stellate-appearing tan material with some red below it. The exact appearance, however, varied considerably. Microscopic examination of the unstained sediment in 0.88 *M* sucrose revealed some erythrocytes and ghosts, numerous particles—apparently mitochondria, large numbers of irregular, roughly spherical or cuboidal granules, many of them in clumps, and a background of unresolved particles. Within 15 minutes after intravenous injection of S^{35}AγG, the specific activity of the TCA precipitate of this Sed fraction was twice and by 24 hours it was 36 times that of the remaining mitochondrial fraction.

Data in table 1 show that although the mitochondrial fraction (M) had 1.7 times the specific activity of the whole homogenate at 1 day, the concentration in the Sed fraction was so high that less than 3% contamination of mitochondria with Sed would account for all of the activity of the former. In fact, by 1 day, the distribution of activity between the pink and tan mitochondria (Mp and Mt) was what might be obtained if the activity of the mitochondrial fraction depended entirely upon particles of Sed which had not settled down to the bottom of the precipitate.

Radioactivity from S^{35}A-stromata was, in general, similarly distributed, but the relative concentration in the Sed fraction was not so high. The ratio of concentration in the Sed to that in the mitochondria 6 to 8 minutes after injection was 1.07 (0.53–1.70) in 5 rabbits, at 1 day in 6 rabbits it was 8.3 (5.0–11.8) and at 3 to 7 days in 3 rabbits it was 13.5 (8.4–17.2). Some of these rab-

TABLE 1.—*Concentration of S^{35} in 7% trichloroacetic acid precipitates of liver fractions* from rabbits injected with radioactive sulfanil-azo-bovine γ globulin.*

	Relative specific activity of fraction†				Percent total S^{35} of whole homogenate (H) in fraction				Percent of total TCA-precipitable material of whole homogenate (H) in fraction‡			
Rabbit no.:	112	94	93	92	112	93	93	92	112	94	93	92
Time killed:	16 min	1 day	7 days	Control§	16 min	1 day	7 days	Control	16 min	1 day	7 days	Control
Fraction:												
N	0.58	0.36	0.38	0.81	18	10	9	22	32	28	23	27
M	2.6	1.7	1.3	0.4	43	23	18	4.5	16.4	13.4	14.1	10.2
M_p	2.8	1.0	0.9		14	4.8	4.8		4.9	4.7	4.7	
M_t	2.5	2.0	1.4		29	18	13		11.5	8.7	9.4	
Sed	4.9	60	40	0.14	4.6	26	21	0.07	0.95	0.42	0.52	0.51
P	1.3	0.2			13	0.6			9.8	2.6		
S_t	0.4	0.6	0.6	1.4	20	29	36	78	47	46	59	53
Total of all fractions					86	88	84	104	96	88	96	91

* Designation of fractions: H, original homogenate; N, nuclear; M, mitochondrial layer; M_p, fluffy pink layer of M; M_t dense tan layer; Sed, compact sediment underneath M_t; P, submicroscopic particles; S_t, supernate from M and washings from all fractions (this fraction includes P).

† Specific activity of fraction/specific activity of H.

‡ Total mg TCA precipitate per g fresh liver in whole homogenate: 16 min, 175; 1 day, 149; 7 days, 128; control, 156.

§ Normal livers mixed with $SA\gamma G$ as described in *Materials and Methods*.

bits had been immunized, splenectomized, or X-irradiated as will be reported elsewhere, but these treatments apparently had no effect on either the gross or subcellular distribution of S^{35}. Thus, the radioactivity from a labeled particulate antigen is also retained in this fraction.

Data in table 1 for the percentage distribution of radioactivity show that by 1 day the Sed fraction might contain more than the whole mitochondrial fraction (M) of the total TCA-precipitable activity from $S^{35}A\gamma G$ remaining in the liver. As compared to the original homogenate the microsome fraction (P) from the liver at 1 day had a specific activity of 0.2 and contained less than 1% of the total activity. Therefore, by 1 day after injection, the Sed fraction comprising about 0.5% of the protein of the homogenate contained the highest concentration and the largest total amount of the protein-bound activity of any liver fraction down to a particle size of 100 mμ or less. In the control experiment this Sed fraction had the lowest concentration and lowest total amount of S^{35} of any of the fractions isolated. Controls for the $S^{35}A$-stromata gave similar results but with this particulate antigen the pink mitochondria (Mp) had a relative activity about 7 times that of the original homogenate. The activity of the combined mitochondrial fraction (M) in the control was thus about twice that of the homogenate, but the activity of both tan mitochondria and Sed were less than one-half that of the homogenate. Therefore, the high concentration of activity in the Sed from livers of injected rabbits was not due simply to nonspecific adsorption or centrifuging out of the antigen in this fraction.

DISCUSSION

In dealing with radioactive labeled antigens the TCA-precipitable radioactivity in the tissues is frequently referred to as "antigen."[1,4] In general, there would seem to be no assurance that determinations of radioactivity from labeled antigens are actually tracing the active antigenic moiety. However, it has been known since the work of Landsteiner[13] that the sulfanil-azo group coupled to foreign protein causes the formation of antibodies in

13. Landsteiner, K. 1947, The Specificity of Serological Reactions, revised ed., Cambridge, Harvard Univ. Press.

rabbits which react specifically with sulfanilic acid. This observation has been confirmed for the SAγG and SA-stromata used in the present studies. Therefore, at least some of the sulfanil-azo groups injected must be part of the active antigen. Some evidence that the S^{35} of $S^{35}A\gamma G$ is metabolically inert and a reliable tracer for the sulfanil-azo group in vivo has already been published.[9] In addition, it has recently been found that S^{35}A-azo-glycyl-tyrosine gave only about 1% as much TCA-precipitable activity in livers or spleens of mice as was found for the intact antigens in rabbits and that $S^{35}A\gamma G$ did not give rise to more than traces of S^{35} in the cystine sulfur of the livers of mice or rabbits.[10] Haurowitz et al[14] have very recently published direct evidence that the S^{35} remaining in rabbits as long as 1 month after injection of $S^{35}A\gamma G$ is mostly present as intact sulfanil-azo-groups. These findings indicate that determinations of TCA-precipitable S^{35} are probably a reliable quantitative measure of sulfanil-azo groups bound to original intact antigen or to fragments of high molecular weight which may be the active antigen in the tissues. Therefore, the sediment fraction described here seems to me to be of considerable importance in the localization of antigens within the tissues and may be of significance for other studies involving tissue fractionation. It seems likely that this fraction may include material similar to the outer layer of the fixed and sectioned mitochondrial pellets in which Gitlin et al[2] observed a high concentration of dye azo-proteins by direct microscopic examination.

14. Haurowitz, F., Walter, H. and Humm, F. D. 1954, Federation Proc. 13: 496.

It is apparent that the biochemical and morphological heterogeniety of the mitochondrial fraction is accompanied by heterogeniety with respect to the localization of antigens. This Sed fraction itself is morphologically very heterogeneous. Some preliminary attempts at subfractionation by differential centrifugation and density gradient centrifugation have been made, but except for small amounts of low activity material, the fractions obtained have not differed in specific activity by more than a factor of 2.

The observations reported here support the contention of Gitlin et al that the label from injected antigens found in the mitochondrial fraction has not yet been proven to be bound to the mitochondria.

SUMMARY

A heavy subfraction from rabbit liver mitochondria has been found to take up and retain a high concentration of radioactivity from the soluble antigen, S^{35}-sulfanil-azo-bovine serum γ globulin, as well as from the particulate antigen, S^{35}-sulfanil-azo-sheep red cell stromata. This fraction which contained less than 1% of the total protein of the liver contained over 20% of the radioactivity from the soluble antigen at 1 and 7 days after injection. Evidence supporting the belief that this radioactivity is a reliable tracer for antigenic material in vivo is discussed.

Since this paper was submitted for publication, Haurowitz (personal communication) has observed by radioautography that the radioactivity of $S^{35}A\gamma G$ injected into rabbits was localized in a narrow layer at the bottom of the mitochondrial layer in sedimented pellets from liver homogenates.

PLAGUE-INFECTED FLEAS FROM NORTHERN NEW MEXICO WILD RODENTS

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The discovery of California ground squirrels infected with *Pasteurella pestis* in the San Francisco Bay area, in 1908, led to extensive investigations by state and federal public health agencies to determine the geographic extent of the infection in wild rodents. As a result of these investigations, plague-infected wild rodents and fleas were first found in New Mexico in 1938.[1] Link[2] described a more recent epizootic in northern New Mexico (Rio Arriba County) in which *Past. pestis* was isolated 18 times from fleas and tissues of whitetail prairie dogs (*Cynomys gunnisoni*) and once each from fleas and lice from yellowbelly marmots (*Marmota flaviventris*). Rodents other than deer mice (*Peromyscus maniculatus*) were scarce; the decreased numbers possibly resulted from plague mortality. Plague organisms were not found in laboratory tests on 467 fleas from 1005 deer mice and these mice seeemed free of the infection at the time of the study. These findings led Link to conclude that prairie dogs formed the primary plague reservoir. Field observations of epizootics, and frequent collection of tissue and fleas infected with plague organisms from ground squirrels, woodrats, and prairie dogs indicated to Eskey and Haas[3] that these rodents constitute important primary reservoirs of infection. That these species of rodents suffer severe mortality in plague epizootics is well known. How other associated rodent species are affected by these epizootics or the part each may play in preserving the infective organisms in the interepizootic periods is much less understood.

The Santa Fe Field Station of the Communicable Disease Center was established in Santa Fe County, New Mexico, to study the ecological relationships of wild rodents and their ectoparasites to diseases, especially plague. Plague infection in Santa Fe County was first reported in a fatal human case which occurred 13 miles southeast of Santa Fe in July 1950. Occasional surveys to locate plague in wild rodents in this area were conducted in 1950–1951 by the New Mexico Department of Public Health, in cooperation with the Communicable Disease Center. From July 1951 to May 1953, 30 locations within 21 miles of Santa Fe were sampled in periodic rodent surveys conducted by the Santa Fe Field Station. *Past. pestis* was not found by laboratory tests of rodent ectoparasites collected in the course of these surveys until April 1951, when plague organisms were isolated from 8 fleas (*Monopsyllus wagneri*) from white-footed mice (*Peromyscus sp.*) which were trapped 6½ miles west of the residence of the fatal human case.

Past. pestis was, again on April 28, 1953, isolated from a pool of 94 fleas (*M. wagneri* and *Orchopeas leucopus*) and unidentified lice collected from 4 white-footed mice (*Peromyscus leucopus*) and 45 piñon mice (*Peromyscus truei*). The plague-positive material was collected one mile north of Lamy, in a mile-long canyon 6600 to 6800 feet above sea level, in the southern edge of

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1. 1938, Pub. Health Rep. **53**: 1628–1629.
2. Link, V. B. 1949, Am. J. Trop. Med. **29**: 493–500.
3. Eskey, C. R. and Haas, V. H., 1940, Pub. Health Bull. no. 254: vol. 83.

the Sangre de Cristo Mountains. The steeply sloping sandstone canyon walls rise 50 to 75 feet from the narrow sandy stream bed which carries water briefly after infrequent summer rains. Three to four miles further north, the Upper Sonoran Life Zone plants and animals are displaced by life more typical of the Transition Life Zone. The most obvious plant life of the arid canyon consists of piñon pine (*Pinus edulis*), one-seeded juniper (*Juniperus monosperma*), and clumps of scrub oak (*Quercus grisea*). Grass of many species and other herbaceous plants grow sparsely. The canyon is located 14 miles south of Santa Fe and 6 miles southwest of the residence of the 1950 human plague case.

METHODS

Following the recovery of plague organisms, a more detailed study of the epizootic area was started May 20, 1953. After the initial survey in April in which the collected rodents were killed by break-back traps, further rodent population disturbance was reduced by the use of live-catch traps. All animals not sacrificed for special studies were released at the place of capture after laboratory examination. Ectoparasites usually were held individually until identified and all of a single species from a single animal were combined for trituration and inoculation into laboratory mice or guinea pigs. When any of the inoculated animals either died or showed evidence of infection within 10 days, its organs were thoroughly cultured and usually reinoculated into guinea pigs. All laboratory tests were performed by the San Francisco laboratory of the Communicable Disease Center.

RESULTS

The total rodent population of the canyon was never trapped, but the various species living there were sampled. The extent of collections, listing both infected and noninfected ectoparasites, are shown in table 1. From the 10 kinds of mammals, 13 species of fleas, 4 of mites and 2 of lice were removed. In spite of the light ectoparasite infestation of *Neotoma albigula*, 9 different species were collected, the largest number of kinds of ectoparasites taken from a single host species. Of the 6 species of fleas taken from *P. truei*, only 2 were commonly present. The most frequently encountered flea, *M. wagneri*, was taken from all 3 species of *Peromyscus* and *Reithrodontomys*. *O. leucopus* was found on 2 *Peromyscus* species and *Reithrodontomys*. In addition to their normal host, one (*Diamanus montanus*) of the two species of ground squirrel fleas was taken twice from mice and the other, *Hoplopsyllus anomalus*, once from *Neotoma albigula*. The decreasing degree of *M. wagneri* infestation of *P. truei* from May through August is shown in table 2. Ectoparasites collected in April are omitted from the table because different collection methods were used after that month.

From May 20 through June 11, 1953, 14 pools of plague-infected fleas were collected from 6 species of rodents (table 3). The epizootic was probably near a peak on May 27, when 6 of the 15 pools of plague-infected fleas were collected. Since fleas were tested in groups of from 1 to 86, absolute numbers of infected fleas were not always obtainable. Most of the pools contained only small numbers of fleas; however, the few larger groups have acted to set the upper limit of the possible number of infected fleas higher than could logically be expected to have actually occurred. At least 3 and not more than 37% of 433 fleas tested (table 3) were infected. A figure between these extremes is indicated by the finding of 14% of the pools to be infected. Of the 6 species of fleas found infected, 2 *Peromyscopsylla hesperomys adelpha* and *Stenistomera* (*Miochaeta*) *macrodactyla* were found naturally infected with plague organisms for the first time.

Study of data on extent of samples (table 1) and distribution of plague-

TABLE 1.—*Number and kinds of ectoparasites and mammals taken from May 20 to August 18, 1953 at Lamy, New Mexico.*

	Hosts								
	Citellus variegatus	*Dipodomys ordi*	*Dipodomys spectabilis*	*Eutamias quadrivittatus*	*Neotoma albigula*	*Peromyscus leucopus*	*Peromyscus maniculatus*	*Peromyscus truei*	*Reithrodontomys megalotis*
Total examinations of rodents	2	6	1	5	71	30	5	191	45
Ectoparasites									
Fleas:									
Anomiopsyllus nudatus					7				
Diamanus montanus	10							1	1
Echidnophaga gallinacea	1				2				
Hoplopsyllus affinis								1	
Hoplopsyllus anomalus	51				1				
Meringis rectus			10						
Monopsyllus eumolpi				1					
Monopsyllus wagneri						72	15	338	17
Orchopeas leucopus						23		195	8
Orchopeas sexdentatus ssp.					8			1	
Orchopeas sexdentatus neotomae					9				
Peromyscopsylla hesperomys adelpha						1	2	1	
Stenistomera (*Miochaeta*) *macrodactyla*					1				
Mites:									
Bdellonyssus bacoti		2				1		12	
Brevisterna utahensis					5				
Haemolaelaps glasgowi	1					3		9	
Hirstionyssus neotomae					1				
Lice:									
Neohaematopinus citellinus	1								
Neohaematopinus neotomae					15				

No ectoparasites were found on 25 *Perognathus flavus*.

infected fleas (table 3) give preliminary indication of the rodents and their ectoparasites most closely associated with spread of the disease. Infected fleas were taken until June 11 from at least 3 individual *P. truei*, 2 *Reithrodontomys*, 2 *N. albigula*, and single *P. maniculatus*, *P. leucopus*, and *Citellus variegatus*. Collections were insufficient to provide evidence for detailed conclusions but data are recorded in the hope that, when combined with future observations, significant associations between plague, rodents, and fleas may be established.

TABLE 2.—*Flea infestation of* Peromyscus truei *at Lamy, New Mexico.*

1953	*P. truei* examinations	*Monopsyllus wagneri* Percent*	*Monopsyllus wagneri* Index†	*Orchopeas leucopus* Percent	*Orchopeas leucopus* Index
May 20–29	62	90	3.3	63	1.1
June 5–12	35	66	1.5	46	1.0
June 18–23	23	61	1.4	56	1.1
June 30–July 10	22	64	1.2	41	0.7
July 17–24	40	22	0.3	33	1.1
August 18	9	22	0.2	44	0.9

* Percent of mice infested with fleas.
† Average number of fleas per mouse examined.

It should be emphasized that finding of plague-infected fleas on a specific animal does not necessarily indicate infection of that animal. The recapture of 4 apparently healthy mice (2 *P. truei* and 2 *R. megalotis*) from 15 to 65 days after they had provided plague-infected fleas may be most readily explained by the fleas acquiring the plague organisms from other rodents. Apparently the fleas were not infective, did not attempt to feed on these hosts, or the hosts were resistant to infection. Although definite conclusions are impossible, it is interesting to note that a rock squirrel, *C. variegatus*, provided plague-infected specimens of *H. anomalus*, but also yielded 10 specimens of *D. montanus* and 1 of *Neohaematopinus citellinus* that were apparently not infected.

In the period that plague-infected fleas were collected, all rodent species were reproductively active and one-quarter of the individuals examined were immature. Lactating females indi-

TABLE 3.—*Species of fleas infected with* Past. pestis *showing percentages of infection by species and host, Lamy, New Mexico, May 20 to June 11, 1953.*

Flea and host	Pools of fleas		Individual fleas	
	Number tested	Percent infected	Number tested	Percent infected*
Monopsylla wagneri ex:				
Peromyscus truei	45	4	258	1 (35)
Peromyscus maniculatus	2	50	4	25
Peromyscus leucopus	8	0	36	0
Reithrodontomys megalotis	3	33	11	9 (55)
Total	58	7	309	1 (32)
Orchopeas leucopus ex:				
Peromyscus truei	28	7	88	2 (44)
Peromyscus leucopus	4	25	7	14
Reithrodontomys megalotis	2	50	2	50
Total	34	12	97	4 (42)
Peromyscopsylla hesperomys sidelpha ex:				
Peromyscus truei	1	100	1	100
Peromyscus maniculatus	2	50	2	50
Peromyscus leucopus	1	100	1	100
Total	4	75	4	75
Orchopeas sexdentatus neotomae ex:				
Neotoma albigula	4	25	7	14 (57)
Stenoistomera (Miochaeta) macrodactyla ex:				
Neotoma albigula	1	100	1	100
Hoplopsyllus anomalus ex:				
Citellus variegatus	1	100	15	7 (100)
Grand Total	102	14	433	3 (37)

* In many cases, represented in this column by two figures on a single line, absolute numbers of infected fleas were unobtainable by reason of having pooled groups of fleas for plague tests. Where two numbers occur on a single line, the first represents percentages based on the assumption that only one flea in each infected pool was infected; the second number, in parentheses, represents the upper limit for percentages of infected fleas, based on the unlikely assumption that all fleas in each infected pool were infected. Where only a single figure is given on a line, it represents a percentage derived from a known number of infected fleas.

cated the presence of additional young confined to nests. The continued appearance of young rodents not having experienced previous infection with plague organisms suggests that the apparent disappearance of plague-infected fleas did not result from the rodent population developing resistance associated with recovery from the infection. Facts regarding inherited resistance in the species of rodents studied are yet to be determined. The disappearance of plague-infected fleas was not correlated with depletion of the rodent population which seemed to remain fairly constant; at least no severe reduction in numbers was detected. The only animal known to have died of plague was a *P. maniculatus* found dead in a live-catch trap. A stained smear of its heart blood contained many bipolar bacilli and *Past. pestis* was isolated from its tissues.

The failure to demonstrate infected fleas taken from the area after June 11 coincided with the gradual reduction of the number of *P. truei* infested with *M. wagneri* (table 2), as well as the beginning of the summer high temperatures. Atmospheric humidity, which was low for the whole period of study, was not apt to be a factor influencing the course of the infection.

The determination of precise geographic limits of an epizootic of short duration presents many difficulties. In this study, the location was known of 8 traps capturing rodents with infected fleas. Six of these locations were in the upper half mile of the canyon and 2 were in the lower half mile. The reason for the concentration of infected fleas in the upper portion of the canyon is not evident, since none of the various species of rodents or fleas were similarly re-

stricted and the populations were sampled fairly uniformly. Plague organisms were not detected in fleas secured from rodents taken in the epizootic period in a trap line extending outward 1 mile from the canyon mouth. Six other locations, 5 to 12 miles from the epizootic area, were sampled in the same period without the detection of *Past. pestis*. The failure to demonstrate plague organisms from these localities suggests that the epizootic was of limited extent.

The epizootic was notable for the complete absence of marmots and prairie dogs, as well as the scarcity of other sciurids (ground squirrels and chipmunks), which are frequently ascribed major roles in North American plague epizootics.[4] This epizootic involved primarily mice (*Peromyscus* and *Reithrodontomys*) and woodrats (*Neotoma*).

The widely distributed and locally numerous mice and woodrats in the areas studied may form avenues for the dissemination of plague. In contrast to mice and woodrats, the prairie dogs, ground squirrels and chipmunks in the studied areas occurred in smaller numbers and had a more discontinuous, spotty distribution which would appear to render them ineffective in spreading the disease to other populations.

4. Meyer, K. F. 1942, Am. J. Trop. Med. 22: 9–36.

SUMMARY

From April 28 to June 11, 1953, plague organisms were demonstrated in ectoparasites from rodents collected in a canyon 1 mile north of Lamy. *Pasteurella pestis* was isolated from 6 species of fleas (*Hoplopsyllus anomalus*, *Monopsyllus wagneri*, *Orchopeas leucopus*, *O. sexdentatus neotomae*, *Peromyscopsylla hesperomys adelpha*, and *Stenistomera* (*Miochaeta*) *macrodactyla* collected from 6 species of rodents (*Citellus variegatus*, *Neotoma albigula*, *Peromyscus leucopus*, *P. maniculatus*, *P. truei*, and *Reithrodontomys megalotis*).

STUDIES ON IMMUNIZATION AGAINST PLAGUE

VIII. STUDY OF THREE IMMUNIZING PREPARATIONS IN PROTECTING PRIMATES AGAINST PNEUMONIC PLAGUE

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While it is generally held that vaccination is of value for controlling insect-borne bubonic plague, most workers in this field doubt or even deny its efficacy in combatting pneumonic plague. This skeptical attitude, taken by several authorities in regard to vaccines made with killed organisms,[1] has been shared by the advocates of vaccination with live avirulent plague bacilli. Otten[2] was so convinced that his Tjiwidej strain conferred no protection against pneumonic plague that he excluded cases of this nature from the statistics he compiled to prove the value of his method. Girard, in a recent summary of his experiences in Madagascar,[3] expressed the opinion that his avirulent E. V. vaccine, while not directly efficacious in the case of pneumonic plague, was of value insofar as it cut short the incidence of bubonic plague which forms the fountainhead of pulmonary infections.

Since no large-scale comparative tests have been made to study the efficacy of the various vaccine preparations in experimental pneumonic plague infection under uniform conditions, it seemed important to embark on a series of such experiments, using, in contrast to most previous workers, the monkey in place of the guinea pig as test animal and paying attention to the prophylactic value of fraction I extracted from the plague bacillus[4] as well as of the usual killed and live vaccines. As a first step it was necessary to study the unimpeded course of experimental pneumonic plague in the monkey. The degree of immunity to intratracheal infection present in monkeys that had recovered from cutaneous bubonic plague was also a subject of preliminary attention.

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1. Report of the International Plague Conference held at Mukden, 1911. Manila, 1912.
2. Otten, L. 1941, A live plague vaccine and the results. Mededeel. v. d. dienst d. volksgezondh. in Nederl.-Indië, **30**: 61–110.
3. Girard, G. 1951, La peste. Contribution apportée par Madagascar à l'état actuel de nos connaissances. Semaine d. hôp. de Paris, **27**: 474–478.

MATERIALS AND METHODS

Three types of vaccines—living avirulent *Pasteurella pestis*, strain E.V. 76; formalin-killed virulent *Past. pestis*, strain Yreka, and fraction I—were used to immunize *Macacus rhesus* monkeys weighing 2 to 5.5 kg. The E.V. 76 was prepared from a 72-hour culture grown on Martin's agar at 26 C and harvested in isotonic saline. Viability estimated by plate count ranged from 43% to 94%, with an average of 75%. Formalin-killed Yreka bacillary suspension (Army general issue) was initially obtained from the Cutter Laboratories. For later studies this vaccine was locally prepared from a 72-hour culture grown at 37 C on beef-heart infusion agar. The bacilli were harvested in isotonic saline and killed with 0.5% formalin. Total bacterial counts of both vaccines were estimated through microscopic observations. Fraction I was extracted from acetone-killed and dried *Past. pestis*, strain Yreka, precipitated and crystallized according to the method of Baker and his associates.[4] This soluble antigen is also present in E.V. 76 and appears to be one of the constituents of the capsule of *Past. pestis*.

Passive immunity studies were made with hyperimmune anti-fraction I rabbit serum, with concentrated antiplague globulin (rabbit, prepared by Lederle Laboratories) and with hyperimmune fraction I antiserum adsorbed with fraction I. The hyperimmune anti-fraction I rabbit serum contained complement-fixing antibody to a dilution of 1:320 and approximately 45 units of mouse-protective antibody.* Concentrated antiplague globulin was made by hyperimmunization of rabbits with living avirulent *Past. pestis*, strain A1122; the serum was then fractionated and concentrated. It contained fraction I complement-fixing antibody to a dilution of 1:1024, agglutinating antibodies to 1:4096 and 450 units of mouse-protective antibody. Repeated precipitation of the anti-fraction I serum with fraction I removed all anti-fraction I antibody, as measured by complement fixation.

Doses of each vaccine and serums are listed later in the text and in table 4. Monkeys were observed for vaccine reactions and elevation in temperature. The only untoward effects of vaccination followed intravenous inoculation with E.V. 76. This was always associated with extensive thrombophlebitis of the vein in which the E.V. 76 was administered.

The highly virulent Indian strain 195/P was the infecting agent in all instances. This organism was grown for 24 hours at 37 C in beef-heart infusion broth. The infective inoculums were suspended in isotonic saline containing 10% monkey serum; from these, plate counts were made immediately before and after instillation to determine the infective dose.

Intratracheal infection was induced with the monkeys under light nembutal anesthesia; the animals were intubated with a laryngoscope and 1 ml of the infective inoculum was delivered through a polyethylene catheter in the trachea.

The rectal temperature of the infected animals was taken twice daily, and their general state of health was noted at the same time. Samples for blood culture were taken daily when the rectal temperature reached 40 C. Frequent roentgen examinations of the thorax were made.

The presence of serum antibodies induced by active immunization was determined 2 to 5 days before intratracheal challenge. Antibodies acquired through passive immunization were measured on serums drawn within an hour of challenge. Complement-fixing antibody was determined by the method of Chen,[5] mouse protective antibody by the method of Meyer and Foster[6] and agglutinins were tested for with washed formalinized suspensions of *Past. pestis* strain 195/P.

The organs of the animals that succumbed were examined grossly and microscopically, and lungs and blood and occasionally other organs were cultured for *Past. pestis*. In all fatalities save one the diagnosis of pneumonic plague was established; the exception was an instance of septicemic plague.

The susceptibility of the rhesus monkey to experimental pneumonic plague and the usual clinical course of the disease was studied in unimmunized animals inoculated intratracheally with varying numbers of *Past. pestis*.

The immunity of monkeys that had recovered from bubonic plague was tested by intratracheal instillation of *Past. pestis* in graded doses. These monkeys had previously been immunized with the avirulent strain E.V. 76 or fraction I and had survived subcutaneous infection with 1,600,000,000 to 2,300,000,000 virulent *Past. pestis* 2 to 5 months prior to intratracheal challenge.

PLAN OF IMMUNIZATION

Each of the three vaccines was evaluated in varying doses and schedules:

Scheme I: dosage recommended for plague prophylaxis in man.

E.V. 76.—A single dose of 1,000,000,000 subcutaneously to 5 monkeys.

Yreka.—A total of 3,000,000,000 organisms, over 8 days, to 5 monkeys (1,000,000,000 on day 1; 2,000,000,000 on day 8, subcutaneously).

Fraction I.—A total of 3.5 mg over 75 days to 5 monkeys (basic series: 1 mg

* We are grateful to Dr. T. H. Chen for carrying out the serologic procedures and plate counts.

4. Baker, E. E., Sommer, H., Foster, L. E., Meyer, E. and Meyer, K. F. 1952, Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J. Immunol. **68**: 131–145.

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6. Meyer, K. F. and Foster, L. E. 1948, Measurement of protective serum antibodies in human volunteers inoculated with plague prophylactics. Stanford M. Bull. **6**: 75–79.

on days 1, 8 and 15; booster, 0.5 mg on day 75, subcutaneously).

Scheme II: short term massive dosage.

E.V. 76—A total of 4,000,000,000 organisms over 10 days to 2 monkeys (1,000,000,000 intravenously on days 1 and 8; 1,000,000,000 intramuscularly on days 9 and 10).

E.V. 76.—A total of 6,000,000,000 organisms over 10 days to 3 monkeys* (1,000,000,000 intravenously on days 1, 2, 3 and 8; 1,000,000,000 intramuscularly on days 9 and 10).

Yreka.—A total of 225,000,000,000 organisms over 16 days to 2 monkeys (25,000,000,000 on days 1, 2, 3, 6, 7, 8, 14, 15, 16, intramuscularly).

Fraction I.—A total of 18 mg over 11 days to 4 monkeys (1 mg intravenously on days 1, 2 and 3; 5 mg intramuscularly on days 9, 10 and 11).

Scheme III: long term massive dosage.

E.V. 76.—A total of 4,000,000,000 organisms over 92 days to 1 monkey (basic series: 1,000,000,000 subcutaneously on day 1; booster: 1,000,000,000 by intratracheal spray on days 78, 84 and 92).

E.V. 76.—A total of 5,000,000,000 organisms over 66 days to 3 monkeys (basic series: 1,000,000,000 subcutaneously on day 1; boosters: 1,000,000,000 intravenously on days 57 and 64; 1,000,000,000 intramuscularly on days 65 and 66).

Yreka.—228,000,000,000 organisms over 88 days to 3 monkeys (basic series: 1,000,000,000 on day 1, 2,000,000,000 on day 8, both subcutaneously; boosters: 25,000,000,000 intramuscularly on days 73, 74, 75, 78, 79, 80, 86, 87 and 88).

Fraction I.—19 mg over 45 days to 4 monkeys (basic series: 1 mg subcutaneously on days 1, 8 and 15; boosters: 1 mg in Bayol-Arlacel† intramuscularly on day 35, 5 mg intramuscularly on days 43, 44 and 45).

Scheme IV: long term moderate dosage.

Fraction I.—5.5 mg over 170 days to 4 monkeys (basic series: 1 mg subcutaneously on days 1, 8 and 15; boosters: 1 mg on day 96, 0.5 mg on days 106, 130 and 170).

Single dose passive immunization, using the intravenous route, was done 6 to 24 hours before challenge. Concentrated antiplague rabbit globulin was injected into 2 monkeys in doses of 1.0 and 10 ml, respectively. Fraction I antiserum, in 10 ml doses, was administered to 3 monkeys. Fraction I antiserum absorbed with fraction I, in 10 ml doses, was given to 2 monkeys.

Immunity was tested 15 to 44 days after the last vaccination by intratracheal instillation of 10,300 ± 1,100 *Past. pestis*. Similarly, monkeys with passive immunity were challenged with 10,300 ± 1,100 *Past. pestis* 6 to 24 hours after intravenous administration of serum. On each occasion unimmunized monkeys were infected intratracheally with 100 to 10,000 *Past. pestis* from the same preparation to demonstrate the virulence of the organism.

RESULTS

The intratracheal instillation of approximately 100 virulent *Past. pestis* was lethal to more than 50% of the unimmunized monkeys (table 1). This was taken as the minimal infective dose. Only 3 of 46 rhesus monkeys inoculated with 1000 or more organisms spontaneously resisted disease. The pattern of disease was established in 17 unimmu-

* These monkeys had previously received 3,000,000,000 formalin-killed Yreka 50 days before; for practical purposes they may be considered in this short term massive dose group.

† The only occasion on which fraction I was not administered in aqueous solution.

TABLE 1.—*Susceptibility of unimmunized monkeys to intratracheal infection with* Past. pestis.

Number of organisms	Survivors	Total
10	2	2
120–270	4	12
800– 2,700	1	18
9,500– 12,000	1	18
120,000–270,000,000	1	10

nized monkeys inoculated with 10,600 ±1,400 *Past. pestis* (table 2), the dose used for challenging immunized animals. The mean survival time from inoculation to death was less than 5 days; in only one instance did an animal survive for 7 days. Daily blood cultures in 14 of this group revealed septicemia on the 1st day of disease* in 5, and in 13 by the 2nd day. In no instance did an unimmunized monkey with roentgen evidence of pneumonia recover. If pneumonia could not be demonstrated by roentgen examination, clinical disease was usually limited to temperature elevation above 40 C for a few hours. Blood cultures were invariably sterile. Agglutinins were present in low titer (1:4 to 1:6) 10 to 26 days later.

Resistance of monkeys that had recovered from bubonic plague was impressive (table 3). All resisted infection with up to 1,730,000 *Past. pestis*. Both of 2 monkeys 2 months past bubonic plague survived challenge with 173,000,000 organisms in spite of demonstrable pneumonia, by roentgen examination.

Results of challenge of immunized monkeys with 10,300±1,100 *Past. pestis* are given in table 4. There was little protection with dosages recommended for antiplague immunization of man. Alteration of the disease was apparent in some of the monkeys that died. In one animal immunized with formalin-killed Yreka, septicemia was not established until the 4th day of disease and the course from infection to death was delayed to 9.5 days. Of 3 monkeys given E.V. 76, 2 had no evidence of antemortem septicemia, and a third had a total course of 10.5 days.

Since the antigenic deficit seemed to be quantitative, rather than qualitative, the protective effects of larger doses were studied. With massive vaccine doses given over a short period the survival rate increased little. Two of the 5 survivors given 4 to 6 billion E.V. 76 over 10 days had pneumonia. One of the 2 animals given 225 billion formalin-killed Yreka over 16 days had a stormy course with pneumonia, but eventually recovered.

Extension of the interval during

* Beginning of disease = first rectal temperature rise to 40 C.

TABLE 2.—*Course of pneumonic plague in unimmunized monkeys inoculated intratracheally with 10,600* Past. pestis.

Monkey no.	Incubation period,* days	Duration of disease,† days	Appearance of bacteremia, day of disease	Total duration (inoculation to death), days
6	2.0	1.5	1.0	3.5
522	1.5	2.5	2.5	4.0
462	2.0	2.5	3.0‡	4.5
484, 490, 497	2.5	2.0	1.0	4.5
741	4.5	2.0	2.0	6.5
735	2.5	3.0	3.0‡	5.5
543, 488, 496	2.5	2.0	2.0	4.5
542	1.5	2.5	2.0	4.0
489	3.5	3.5	§	7.0
506	2.5	3.5	1.0	6.0
523	3.0	2.5	2.0	5.5
526	1.0	3.5	3.0	4.5
527	2.5	3.0	2.0	5.5
Averages	2.6	2.4	1.8	4.9
Maximums	4.5	3.5	2.5	7.0

* Time between inoculation and first temperature of 40 C or more.
† Time between first temperature of 40 C and death.
‡ Blood culture not made earlier in disease (included in average, not in maximum).
§ Blood culture not made after 1st day of disease.

TABLE 3.—*Susceptibility of monkeys recovered from cutaneous bubonic plague to intratracheal infection with* Past. pestis.

Number of organisms	Survivors	Total
1,300	4	4
13,000	4	4
1,730,000	4	4
173,000,000	2*	4
270,000,000	0	7

* The 2 survivors were 2 months past bubonic plague, the 2 that died were 5 months past bubonic plague.

TABLE 4.—*Resistance of immunized monkeys to intratracheal challenge with 10,000* Past. pestis.

Immunizing preparation, dose, duration, number of boosters	Survivors/total	Modified disease* and death	Pneumonia and survival	Unimmunized monkey titration†	
				Challenge, dose, number of *Past. pestis*	Survivors/total
Scheme I: dosage schedule recommended for man					
E.V. 76, 1 billion, 1 day	1/5	3	0	115	2/4
Yreka, 3 billion, 8 days	0/5	1	0	1,145	0/5
Fraction I, 3.5 mg, 15 days, 1 booster	2/5	0	2	11,450	1/5
Scheme II: short term massive dosage					
E.V. 76, 4–6 billion, 10 days	3/5	2	2	112	1/2
Yreka, 225 billion, 16 days	2/2	0	1	1,115	0/1
Fraction I, 18 mg, 11 days	1/4	2	0	11,150	0/3
Scheme III: long term massive dosage					
E.V. 76, 4–5 billion, 66–92 days, 3–4 boosters	4/4	0	1	83	0/2
Yreka, 228 billion, 88 days, 9 boosters	3/3	0	0	900	0/2
Fraction I, 19 mg, 45 days, (including 1 mg in Bayol-Ariacel), 4 boosters	4/4	0	0	9,200	0/2
	4/4	0	0		
Scheme IV: long term moderate dosage					
Fraction I, 5.5 mg, 170 days, 4 boosters	4/4	0	0		

* Modified disease: delay in appearance of septicemia beyond the 3rd day of disease, or a total course of more than 7 days from infection to death.
† Unmmunized monkey titration as that of scheme III.

which vaccination took place provided a more effective immunity. In none of the 4 animals given 19 mg of fraction I over 45 days was there roentgen evidence of pneumonia. Only 1 given 4 to 5 billion E.V. 76 over 66 to 92 days had pneumonia. There was no evidence of disease in the 3 animals given 228 billion formalin-killed Yreka over 88 days. As small a dose as 5.5 mg of fraction I given over 170 days enabled all 4 monkeys to survive challenge without clinical evidence of disease.

Passive transfer of antibodies to fraction I or to whole *Past. pestis* (concentrated antiplague serum) was also effective in either modifying or preventing pneumonic plague (table 5). Administration of 1 ml of concentrated plague antiserum prolonged in one instance the course from inoculation to death to 14 days; blood cultures were sterile for the first 9 days of disease; 10 ml protected another monkey against death, even though pneumonia was apparent on a roentgenogram. Ten milliliters of fraction I antiserum prevented the appearance of clinical signs in 2 monkeys and prolonged the course of illness to 7.5 days from inoculation to death in a third animal.

In striking contrast to these results,

TABLE 5.—*Susceptibility of passively immunized monkeys to intratracheal challenge with 10,000* Past. pestis.

Immunizing preparation	Total dose, ml	Total monkeys	Results		
			Modified disease* without survival	Pneumonia and survival	Survivors/total
Concentrated antiplague serum (Lederle)	1	1	1	0	0/1
	10	1	0	1	1/1
Anti-fraction I serum	10	3	1	0	2/3
Anti-fraction I serum adsorbed with fraction I	10	2	0	0	0/2

Five controls were given 10,400 organisms; none survived.
* Modified disease = delay in appearance of septicemia to the 3rd day of disease or more or a total course of more than 7 days from infection to death.

fraction 1 antiserum from which the antibodies to fraction I had been precipitated showed no ability to protect monkeys against intratracheal inoculation or even to modify the disease.

In general, resistance of monkeys to pneumonic plague was associated with measurable serum antibodies to *Past. pestis* at the time of challenge. Monkeys with any two of the following three antibody levels at the time of challenge survived with but one exception:

Complement-fixing antibody	1:4 or more
Agglutinins	1:16 or more
Mouse-protective antibody	Index of 9 or less

However, monkeys with little antibody measurable by these methods not infrequently survived challenge. This was particularly true of those immunized with E.V. 76 vaccine.

DISCUSSION

Resistance to intratracheal instillation of 10,000 virulent *Past. pestis*, and consequently the prevention of pneumonic plague in the primate host, has been achieved by vaccination with E.V., formalin-killed Yreka or fraction I. Unimmunized rhesus monkeys were highly susceptible to pulmonary infection with *Past. pestis;* as few as 100 organisms inoculated intratracheally caused fatal pneumonic plague in more than 50%.

Protection against pneumonic plague was realized only through repeated immunization, properly spaced, with each of the vaccines tested. The total vaccine dose was of much less importance. Eighteen mg of fraction I given over 11 days protected but 1 of 4 monkeys; 5.5 mg given over 170 days protected all of 4, with signs of disease in none. Four billion to 6 billion E.V. over a 10-day period prevented pneumonic plague in only 1 to 5 monkeys, although 2 more had the disease and survived; the same dose over 66 to 92 days resulted in survival of all of the 4 monkeys immunized, with pneumonia in only 1. Doses and schedules of vaccination with E.V., formalin-killed Yreka or fraction I recommended for the prevention of bubonic plague in man were not effective in preventing experimental pneumonic plague in monkeys. Nevertheless, development of an immunity inadequate in terms of ultimate survival did modify the clinical disease in some instances.

Previously immunized monkeys that recovered from bubonic plague showed a high order of resistance to intratracheal challenge. Evidently the bubonic disease supplied the antigenic mass and duration of stimulation necessary to confer immunity.

Both active and passive immunization studies demonstrated that fraction I stimulated the production of protective antibodies. The important role played by this antigen in conferring immunity against plague has been confirmed by the observation that the removal of antibodies to Fraction I from anti-fraction I serum caused complete loss of protective power. It is the fraction I antigen that is common to all three vaccines tested.

It is concluded that a vaccine to be effective in modifying or preventing pneumonic plague in man must contain adequate fraction I, or in the case of the living avirulent vaccines, have the ability to produce sufficient fraction I in vivo. In addition, immunizations must be spaced over properly long intervals and the initial immunity must be reinforced by subsequent booster injections.

SUMMARY

Experimental pneumonic plague is easily reproducible in *Macacus rhesus*. It was prevented by immunization with

living avirulent *Pasteurella pestis* (strain E.V. 76), formalin-killed virulent *Past. pestis* (strain Yreka) or fraction I, if repeated vaccinations were given. Spacing of individual inoculations and the total period of vaccination were more important than the total dose of the immunizing agent. Doses and schedules of vaccination with these agents usually recommended for antiplague immunization of man did not protect monkeys against pneumonic plague. Antibodies stimulated by fraction I immunization were completely protective.

STUDIES ON IMMUNIZATION AGAINST PLAGUE

XI. A STUDY OF THE IMMUNOGENICITY AND TOXICITY OF ELEVEN AVIRULENT VARIANTS OF VIRULENT STRAINS OF *Pasteurella Pestis*

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Many pathogenic microorganisms undergo spontaneous or artificially induced dissociation into avirulent strains. Various workers studying *Pasteurella pestis* have used several methods to produce avirulent variants of this organism: (a) repeated subcultivation at weekly intervals,[8–10,12,13,15,18,21,22,24] (b) prolonged subcultivation on broth containing alcohol;[5,6,11,13] (c) passage of strains through immune animals[2,13] (d) cultivation in the presence of bacteriophage[14,19,20] or (e) aeration of broth cultures.[7] It seems, however, that the artificial production of avirulent plague strains would be likely to be accompanied by alteration of the bacillus or by loss of much of its envelope substance, which, according to the research of Schütze,[23] Meyer and his collaborators[3,16,17] and Amies[1] is of paramount importance in immunization against plague. Therefore, in the search for immunogenic avirulent *Past. pestis* strains it was considered preferable to use, in place of artificial procedures, the method of single colony picking. As shown by observers such as Otten[18] and Jawetz and Meyer,[13] this method permits isolation of avirulent colonies from primarily virulent strains. The present study is concerned with the isolation of a number of such avirulent colonies and with their immunogenicity and toxicity.

MATERIALS AND METHODS

Parent strains.—Twenty of the *Past. pestis* parent strains used for this study were from the collection of the Hooper Foundation and the cultures ranged in age from 4 years, 2 months to 10 years, 3 months. The remaining 7 parent strains were received from the Laboratory of Hygiene, Kamloops, Canada, through the courtesy of Dr. Francis A. Humphreys, and the cultures ranged in age from 3 years to 11 years. The Hooper strains had been preserved in deep agar or on blood agar slants, the Canadian strains, in hormone agar.

Isolation of avirulent colonies from virulent parent strains.—One loopful of culture was suspended in 0.5 ml of hormone broth in a test tube, and 1 loopful of this suspension was streaked out on a blood agar plate. After 48 hours incubation at 37 C, 10 colonies were picked at random under a dissecting microscope and transferred individually to separate hormone broth tubes. After 24 hours incubation at 37 C, 10^{-5} dilutions were made with each of the latter cultures, and 0.2 ml of these dilutions was injected intraperitoneally into 2 mice each. If the 2 mice survived for 2 weeks, 5 more mice were injected with the material to confirm the loss of virulence. Finally, 2 guinea pigs were inoculated intraperitoneally with 1.0 ml of a 10^{-3} dilution of the strain, after subcultivation in hormone broth for 24 hours at 37 C.

RESULTS

Percentage of avirulent variant colonies isolated from parent cultures.—The percentage of colonies of avirulent strains

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TABLE 1.—*General history of the virulent parent strains and percentage of avirulent colonies isolated.*

Virulent parent culture				Proportion of avirulent colonies, %*
No.	Source	Medium	Age of cultures	
F5513	Flea	Blood agar slant	7 yr, 5 mo	0
F7348	Flea		6 yr, 5 mo	0
F106	Flea		8 mo	0
F4102	Flea		8 yr, 3 mo	0
F957	Flea		10 yr, 3 mo	0
0-148-2	Rat		10 yr	0
Shasta H-2-41	Human		6 yr, 8 mo	0
Hawaii 193	Rat		4 yr, 2 mo	0
B303	Flea	Deep agar	4 yr, 6 mo	0
B391	Flea		4 yr, 6 mo	0
B2767	Flea		4 yr, 4 mo	0
B302	Flea		4 yr, 7 mo	0
B1978	Flea		4 yr, 6 mo	0
B831	Squirrel		4 yr, 6 mo	0
B741	Flea		4 yr, 5 mo	40
B2764	Flea		4 yr, 4 mo	90
B1456	Flea		4 yr, 8 mo	40
B868	Flea	Blood agar slant	4 yr, 6 mo	30
F7793	Flea		6 yr, 6 mo	40
Egypt-53	Human		5 yr	100
P1	Flea	Hormone agar	8 yr	100
P30	Squirrel		8 yr	100
P13	Flea		8 yr	100
P45	Flea		5 yr	50
P39	Unknown		11 yr	100
P51	Flea		3 yr	8
P56	Flea		3 yr	0

* Ten colonies of each strain were tested.

isolated from the 27 parent cultures under study is shown in table 1, together with all data relevant to the parent cultures. Of the 11 cultures preserved on blood agar slants, varying in age from 4 years, 2 months to 10 years, 3 months, only 3 parent strains yielded avirulent colonies, and of the 9 cultures preserved in deep agar for from 4 years, 4 months to 4 years, 8 months, another 3 produced avirulent colonies. On the other hand, of the 7 strains preserved on hormone agar, those 5 or more years old produced a very high percentage of avirulent colonies, while the 2 cultures preserved for only 3 years on hormone agar yielded no avirulent colonies.

Immunogenic power of the avirulent variants.—Although the virulent parent cultures of the newly isolated variants were known to be immunogenic, the immunogenicity of the variants had to be determined by mouse and guinea pig tests. A culture of one variant strain from each of the original virulent cultures, containing living organisms in dilutions of 10^{-1} to 10^{-6}, was inoculated subcutaneously or intraperitoneally into groups of 6-week-old mice of the ABC strain (table 2). For the first 7 strains in the table each dilution was given to 5 mice; for the remaining 5 strains, 10 mice per dilution. A classical avirulent strain, E.V. 76, kindly provided by Dr. G. Girard of the Institut Pasteur, Paris, was included for comparison. Two weeks after immunization all mice were injected subcutaneously with 1 standard infecting dose containing from 100 to 1000 LD_{50}'s of the virulent Shasta strain of *Past. pestis* grown in hormone broth. Animals dying after challenge were autopsied to confirm that their deaths were due to plague infection.

Of the 11 strains tested in mice, strain B741-10-1 had high protective power, but was only partially avirulent, killing several mice immunized with more than 21,000 organisms. The survivors were rechallenged later and the PD_{50} given is based on the results of the second challenge. Of the remaining 10 strains, all proved to be truly avirulent, even when more than 2,000,000 living

TABLE 2.—*Titration of the immunogenic potency of 12 avirulent* Past. pestis *strains for mice (ABC strain).*

Strain and fraction I content	Immunization, number of living organisms in 10^{-1} dilution	Approximate PD_{50}*	
		Subcutaneous	Intraperitoneal
B1456-4 (15%)	1,400,000	21	14
F7793-10 (11%)	1,800,000	58	58
B741-10-1	2,100,000	<21	210
E.V. 76 (1.8–5%)	1,740,000	3,600	2,300
B868-8 (7.5%)	1,000,000	6,000	1,800
B2764-6 (15%)	1,100,000	700	14,000
Egypt 53-1 (8.8%)	1,500,000	900	37,000
P39-9	1,120,000	280,000	2,250
P1-10	2,400,000	170,000	1,300,000
P13-6	1,160,000	>1,160,000	>1,160,000
P45-7	2,440,000	>2,440,000	900,000
P30-3	2,700,000	>2,700,000	770,000

* Values calculated according to Reed and Muench.

organisms were injected. Of these strains the most immunogenic were strains B1456-4, with a subcutaneous PD_{50} of 21 and an intraperitoneal PD_{50} of less than 14 organisms, and F7793-10, with a PD_{50} of 58 organisms for either route. The strains of lowest immunogenicity were the P strains, which in several cases failed to protect 50% of the mice immunized with the greatest number of organisms. Most of these P strains had produced 100% avirulent colonies when streaked on blood agar plates and presumably had lost much of the envelope antigen. The only exception in this group was strain P39-9, with an intraperitoneal PD_{50} of 2250.

The protective power of the 7 strains most immunogenic for mice was also titrated in guinea pigs. The animals were injected subcutaneously with varying numbers of organisms, and their immunity was challenged by the subcutaneous injection of about 1,000,000 bacilli of the Shasta strain. All animals dying after challenge were autopsied to determine the cause of death. The results (table 3) were very similar to those of the mouse protection test.

TABLE 3.—*Titration of the immunogenicity of 7 avirulent* Past. pestis *strains for guinea pigs.*

Immunizing strain*	Approximate PD_{50}†
B1456-4	<50
B741-10‡	<50
P39-9	4,800
F7793-10	5,000
B868-8	5,000
Egypt 53-1	∞ 50,000
B2764-6	1,500,000,000

* Strains B1456-4, B741-10, F7793-10, B868-8, Egypt 53-1 and B2764-6 were tested undiluted and in dilutions of $10^{-5.5}$, $10^{-6.5}$ and $10^{-7.5}$; in most cases 6 animals were used per dilution. Strain 39-9 was tested in dilutions of $10^{-5.5}$ through $10^{-7.5}$. The undiluted culture used for immunization contained 3,000,000,000 organisms; the $10^{-5.5}$, 5,000,000. All of the 6 challenged controls died.

† Challenge: 1,000,000 virulent *Past. pestis*, Shasta strain.

‡ This strain was only partially avirulent in the initial immunization series (10 animals per dilution); the PD_{50} represents the result of the second challenge.

Serologic tests with immune guinea pig serums and hyperimmune chicken serums.—Several of the isolated avirulent strains and 2 control strains, Harbin and E.V. 76, were injected subcutaneously into groups of 4 guinea pigs each, in a dose of 500,000 organisms (total count). Animals were bled 3 weeks after immunization and their serums were tested for agglutination with a standard virulent Yreka strain and for complement fixation with fraction I[4] (table 4). In con-

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firmation of the results of the mouse and guinea pig protection tests, serums of animals immunized with strains B1456-4 and F7793-10 had high agglutinating and complement-fixing titers, while titers of serums of animals given other strains were fairly or very low.

In addition to serologic tests with guinea pig immune serums, cross-agglutination tests were performed with serums of chickens hyperimmunized with the avirulent strains 14, A1122, Harbin and Tjiwidej, all 4 serums having titers of at least 1:160 against their specific antigens. Previous investigations had shown that strain 14 contains little envelope antigen, whereas strains A1122, Harbin and Tjiwidej have a fairly wide envelope[17]. As shown in table 5, the cross-agglutination titers of strain 14 antiserum with all the antigens tested were rather low. On the other hand, these titers of strains

TABLE 4.—*Agglutination and complement fixation test with guinea pig serums.*

Serum of strain	Guinea pig no.	Titers	
		Agglutination	Complement fixation
Harbin	1	1:16++++	1:2++++
	2	1:8++++	1:2++++
	3	1:4++++	1:2++++
	4	0	0
E.V. 76	1	1:2++++	1:2++++
	2	0	0
	3	1:4±	0
	4	1:4±	0
F7793-10	1	1:64++++	1:32++++
	2	1:32++++	1:32++++
	3	1:32++++	1:16++++
	4	1:128++++	1:64++++
B2764-6	1	1:32++++	1:8++++
	2	1:2++++	1:2++++
	3	1:4++	1:2+++
	4	1:4++	1:2++
Egypt 53-1	1	1:4±	1:2+
	2	1:2++++	1:4++++
	3	1:4++	1:2++++
	4	1:4±	1:2+
B868-8	1	1:4++++	1:4++++
	2	1:4±	1:2+
	3	1:4±	1:2+
	4	1:4±	1:2+
B1456-4	1	1:32++++	1:16++++
	2	1:32++++	1:16++++
	3	1:8++++	1:4++++
	4	1:32++++	1:16++++
Control		0	0

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TABLE 5.—*Cross agglutination test with hyperimmunized chicken serums.*

Antiserum	Fraction I content of strain, %	Antigen	Agglutination titer
Anti-14 serum	0.02	14	1:320++++
		B741*	1:10+++
	7.50	B741-10†	1:10+++
	7.50	B868-8	1:10+++
	8.80	Egypt 53-1	1:10++
	15.00	B2764-6	1:10+++
	11.00	F7793-10	1:10+++
	15.00	B1456-4	1:10++
Anti-A1122 serum	15.00	A1122	1:160++++
		B741	1:160++++
		B741-10	1:160++++
		B868-8	1:10+++
		Egypt 53-1	1:20++++
		B2764-6	1:20++++
		F7793-10	1:20++++
		B1456-4	1:40++++
Anti-Harbin serum	3.30	Harbin	1:320++++
		B741	1:320++++
		B741-10	1:320++++
		B868-8	1:20++++
		Egypt 53-1	1:40++++
		B2764-6	1:20++++
		F7793-10	1:20++++
		B1456-4	1:20++++
Anti-Tjiwidej serum	15.00	Tjiwidej 48	1:160++++
		B741	1:80++++
		B741-10	1:160++++
		B868-8	1:80++++
		Egypt 53-1	1:40++++
		B2764-6	1:40++++
		F7793-10	1:20++++
		B1456-4	1:40++++

* Virulent parent strain, fraction I content not determined.
† Partially avirulent.

A1122, Harbin and Tjiwidej antiserums with the virulent strain B741 and the partially avirulent strain B741-10 were high, and they were moderately high with the other antigens tested.

Toxicity of the avirulent strains isolated.—In order to test the toxicity of the avirulent variants, hormone broth cultures grown at room temperature for 19 days were passed through Seitz filters, and 0.5 ml of several dilutions of the filtrates were injected intravenously into mice of the Hamilton strain. The results are presented in table 6. The toxin LD_{50} of the different strains ranged from 0.00625 ml with strain B741-10 (only partially avirulent) to 0.022 ml with strain F7793-10. Strain B1456-4, the most immunogenic of the avirulent group, was also the most toxic, with an LD_{50} of 0.0086 ml. B2746-6 was relatively poor immunogenically, but had a toxin LD_{50} of 0.0089 ml. Strain F7793-10, on the other hand, had a toxin LD_{50} of 0.022 ml and was immunogenic.

DISCUSSION

Study of 11 avirulent strains isolated from old plague cultures of virulent strains yielded data concerning some factors related to spontaneous dissociation of this organism and indicated the set of conditions likely to produce immunogenic avirulent strains. The relationship between the host from which the parent strain was isolated and dissociation to avirulence was difficult to discern because the number of human, rat and squirrel hosts was too small to allow significant comparison. The relationship of culture medium and age of the parent culture was more apparent. Culture medium appeared to be the primary factor in dissociation and age of the culture was an important contributing factor. If the parent strain had been stored on hormone agar slants the percentage of subsequent avirulent colonies was very high. Of 7 virulent strains stored on hormone agar, variants were not isolated from 2 strains stored for 3 years, 50% of the colonies derived from 1 strain stored for 5 years but 100% of those isolated from 4 strains stored for 8 to 11 years were avirulent. On the other hand, 10 of 16 strains stored on blood agar slants or in deep agar for 5 to 7 years yielded no avirulent variants and the remaining 6

TABLE 6.—*Toxicity test of the avirulent strains on mice (Hamilton strain).*

Strain*	LD_{50}	
	Dilution	Ml
F7793-10	1:22	0.022
B868-8	1:46	0.0108
14	1:49	0.01
Egypt 53-1	1:49	0.01
B1456-4	1:58	0.0086
B2764-6	1:56	0.0089
B741-10	1:80	0.00625

* Serial dilutions of 1:10 through 1:160 were given; 0.5 ml of each dilution was injected into 6 mice.

strains yielded an average of 50% avirulent variant colonies. Neither were avirulent variants isolated from 4 strains stored on blood agar for 8 years or longer.

It is often necessary to store virulent plague cultures many years and this study suggests, if maintenance of virulence is sought, the desirability of adding blood or serum to the agar medium and the use of deep stab cultures, rather than surface cultures, when storing the organisms for long periods. Since the tendency to dissociate to avirulence may be due partly to oxidation, sealing culture tubes under vacuum or replacing air with dry nitrogen might aid in maintaining virulence of a culture.

The most satisfactory avirulent strains, from the standpoint of immunogenicity, as determined by mouse and guinea pig protection and serologic tests, were isolated from virulent cultures stored on blood agar or in deep agar for 5 or 6 years. Two strains in particular were highly immunogenic. One strain, B1456-4, stored in deep agar for 4 years, 8 months, had a PD_{50} for mice of from 14 to 21 organisms and for guinea pigs of less than 50 organisms. This strain had a fraction I content of 15% (per 1 mg of dried bacilli) and stimulated the production of a significant anti-fraction I antibody reaction. Of 4 guinea pigs immunized with a single dose of 500,000 organisms (total count), the serum of 3 had agglutination titers of ++++ at 1:32 dilution and complement fixation titers of ++++ at 1:16. Titers for the other animal were ++++ at a dilution of 1:8. Another immunogenic strain was F7793-10, stored on blood agar for 6 years, 6 months. Its fraction I content was 11% and, although it was somewhat less protective than B1456-4, it provoked even better serologic responses, one serum having an agglutination titer of 1:128 and a complement fixation titer of 1:64.

The presence of a relatively large amount of fraction I in the protective strains was confirmed by cross-agglutination tests with serums of chickens hyperimmunized with avirulent strains 14, A1122, Harbin and Tjiwidej. The specific agglutination of serum 14 with its own antigen was considered somatic, since the amount of fraction I in the envelope of strain 14 is so small as to stimulate very little anti-fraction I antibody reaction, even in a hyperimmunized animal. The failure of all the new strains to agglutinate with serum 14, while cross-reacting with the other serums, confirms the reliability of the agglutination test in revealing a fraction I antigen-antibody reaction, since strains A1122, Harbin and Tjiwidej all contain a fairly large amount of fraction I.[17] However, while this test did reveal the presence of soluble envelope antigen in the new strains, it did not reveal the amount, since excess fraction I is removed from the cells by washing in the preparation of the antigen for the agglutination test.[4] The titers of all the strains with different serums were very similar because the amount of fraction I remaining on the washed cells was probably about the same with all the antigens tested except strain 14.

Of the newly studied strains the most toxic was the partially virulent strain B741-10. The most toxic of the avirulent strains was the highly immunogenic strain B1456-4. The next most immunogenic strain, F7793-10, was also the least toxic. Strain B2764-6, a relatively poor immunogenic strain, was next to B1456-4 in toxicity. Toxicity was apparently not related to immunogenicity.

SUMMARY

By the method of single colony picking, 11 avirulent variant strains were

isolated from old cultures of 27 virulent *Pasteurella pestis* strains. The most nearly complete dissociation to avirulence was among cultures preserved on hormone agar for from 5 to 11 years. The most immunogenic variants were derived from strains preserved in deep agar or on blood agar slants for 5 or 6 years. Storage in deep agar or on blood agar slants appeared to be the most satisfactory means of maintaining virulence of a strain for long periods.

Of the 11 variant strains, 10 were completely avirulent and 1 was partially avirulent. Two avirulent strains, B1456-4 and F7793-10, gave excellent protection in both mice and guinea pigs, demonstrated by challenge of immunized animals and by agglutination and complement fixation tests with the serums of immune guinea pigs, and appear to have excellent potentialities as avirulent vaccines, as effective as, or perhaps more so than, E.V. 76 or Tjiwidej. Four other avirulent strains were fairly immunogenic, but stimulated rather weak antibody response in immunized guinea pigs. Four strains were nonimmunogenic.

The immunogenic strains had fairly wide envelopes, as shown by fraction I determination and by cross-agglutination tests with serums of chickens hyperimmunized with 4 established avirulent strains.

Immunogenicity was not apparently related to toxicity.

THE INFLUENCE OF PERTUSSIS VACCINE ON HISTAMINE SENSITIVITY OF RABBITS AND GUINEA PIGS AND ON THE BLOOD SUGAR IN RABBITS AND MICE

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In 1948, Parfentjev and Goodline reported that mice injected with large doses of pertussis vaccine developed a marked increase in susceptibility to histamine. Since this report, the phenomenon has been observed by a number of investigators (Halpern and Roux, 1949; Pittman, 1951a; Thiele and Schuchardt, 1952; Malkiel and Hargis, 1952a; and others). After inoculation of minimal effective amounts of vaccine, the sensitization is demonstrable by the end of 2 days, the maximum degree is usually reached in 4 or 5 days, and by the end of 14 days it is generally much diminished (Pittman, 1951b). However, after intranasal inoculation of living *Haemophilus pertussis* (*Bordetella pertussis*, Moreno-López, 1952), Pittman (1952c) found that (1) the maximum sensitization was reached in 10 to fifteen days, the time was influenced by the size of the infecting inoculum, and that (2) sensitization persisted for several weeks after the bacteria could no longer be recovered from the lungs. Attention was directed to a parallelism between the course of sensitization in the respiratory-infected mice and the time of onset and duration of paroxysmal coughing in the child suffering from pertussis.

Interest in this phenomenon was aroused as a possible clue to some of the unsolved problems in pertussis and pertussis immunization of children. Namely, (1) the peculiar kind of cough which develops after the acute catarrhal stage and persists for weeks after the bacteria can no longer be recovered, (2) the encephalitic symptoms which may occur during the disease, especially in young infants, and (3) the encephalopathies that occur in rare instances after injection of pertussis vaccine. The mouse, however, differs from the child in its physiological response to histamine: it is naturally relatively resistant in contrast to the high degree of sensitivity in the child. It therefore seemed indicated to investigate if animals naturally sensitive to histamine would develop an increase in sensitivity after an injection of pertussis vaccine. The results obtained with rabbits and guinea pigs are given in this paper, with some additional observations on mice.

It was found that both the rabbit and the guinea pig, instead of developing an increase in histamine sensitivity, became slightly but significantly less sensitive. In the preliminary experiments with rabbits when no increase was observed, it was thought that the 5-day interval after the injection of the vaccine might not be the optimal time for observation. Because of the large number of rabbits that would be necessary to investigate other time intervals, it was decided to observe the blood sugar levels after injection of the vaccine as a possible indicator of increased histamine sensitivity. Parfentjev and Schleyer (1949) had reported that mice show a decrease in blood sugar that paralleled the increase in histamine

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sensitivity. In the present work, the observations of these workers with mice were confirmed, but with rabbits there was no decrease in the blood sugar except after repeated intravenous injections of large doses of vaccine that were lethal for about 50% of the animals. At no time did the histamine sensitivity of vaccinated rabbits exceed that of the controls. In additional experiments with mice in which freshly prepared toxic and detoxified vaccines were used, an additional decrease in blood sugar induced by the toxic vaccine was not accompanied by a further increase in histamine sensitivity.

METHODS

Female animals were used, as Pittman (1951a) had found that female mice, both normal and vaccinated, were more sensitive to histamine than male mice. Litter mates as far as possible were paired in the control and the vaccinated groups of the rabbits and of the guinea pigs. At the beginning of an experiment, the rabbits of the New Zealand White strain were 4 to 5 weeks old and weighed between 500 to 700 g, the guinea pigs of the Hartley strain were about 2 weeks old and weighed between 150 and 200 g, and the mice of the NIH BS strain were about 4 weeks old and weighed 12 to 14 g.

The National Institutes of Health reference pertussis vaccine lot no. 4 containing 20,000 million bacteria per ml was used, except as designated in two experiments with mice. Histamine sensitivity was determined 5 days after vaccination unless otherwise indicated.

The histamine given as histamine diphosphate (36.18% histamine base) was administered in relation to the weight of the individual animal, and the doses were expressed in milligrams of the compound per kilogram animal weight. The volume for rabbits and guinea pigs was 0.1 ml per 100 g and for mice 0.1 ml per 10 g. Mice were injected intraperitoneally and rabbits and guinea pigs intravenously. In the animals of the latter two species the reaction began within one minute. After a short stage of excitement, the animals showed blue discoloration of the eyes and ears, the respiration rate dropped and the muscle tonus decreased. In severe reactions, convulsions occurred also within one minute, frequently followed by death immediately or in a few minutes. In preliminary experiments intraperitoneal injections of rabbits and guinea pigs were tried but fatal reactions were irregular even with increased doses of histamine.

The blood sugar determinations were done by the micro method of Folin and Wu. With rabbits all tests were done in duplicate. With mice equal amounts of blood from three mice were pooled for a determination. Food was withheld from the animals for 6 hours before blood withdrawal, except as designated in one experiment with rabbits and a portion of one with mice.

EXPERIMENTAL

After the first histamine sensitivity experiment with rabbits failed to show an increase in sensitivity 5 days after the injection of the vaccine, other rabbits were tested from time to time for histamine sensitivity. Since some of these were from the blood sugar determination experiments, a summary of the results obtained with all the rabbits tested for histamine sensitivity will follow the presentation of the blood sugar determination findings.

The influence of pertussis vaccine on the blood sugar level in rabbits and in mice.—Before undertaking the study with rabbits, an experiment was done to see if the work of Parfentjev and Schleyer (1949) on the relation of blood sugar levels and histamine sensitivity in mice could be confirmed. The confirmatory results obtained are given in table 1. It is shown that 5 days after injection of the vaccine there was a decrease in blood sugar and an increase in histamine sensitivity and that the intensity of each reaction was in direct relation to the amount of vaccine injected.

Three experiments with rabbits were done. In the first one, 14 female rabbits, 2 from each of 7 litters, were used. One animal from each litter was injected intraperitoneally with 40,000 million bacteria of the vaccine, and the other animal from each litter was used as a control. Blood sugar determinations of

TABLE 1.—*Blood sugar levels and histamine sensitivity of mice 5 days after intraperitoneal injection of pertussis vaccine.*

Vaccine dose	Blood sugar level—mg %		Histamine diphosphate-50 mg/kg	
Number bacteria $\times 10^9$	Average*	Range	D/n†	Percent
None	166	218-143	3/22	13.6
2.5	138	182-118	18/26	69.2
20.0	113	145- 84	14/14	100.0

* 36 mice per group. Equal amounts of blood from 3 mice were pooled for a determination.
† Histamine deaths/number injected (survivals after withdrawal of blood from heart for sugar determination).

the 14 rabbits were made daily for 11 days. The results are given in figure 1. Each value given is the average of the duplicate tests per animal. It may be seen that during the 11-day period, the blood sugar levels in the vaccinated rabbits were not different from those in the nonvaccinated control rabbits. The vaccinated rabbits were given two more injections of vaccine with intervals of about 14 days between each of the three injections. Blood sugar determinations were made daily for 4 days after the second injection and for 3 days after the third injection. Again, no change in blood sugar content was observed.

In the second experiment, the rabbits were given the vaccine intravenously, the route that is used for induction of antipertussis serum in these animals. A dose near the borderline of tolerance was selected, as Parfentjev and Schleyer had used a similar one for mice. This dose, determined in a preliminary test, was 20,000 million bacteria. Sixteen rabbits were injected with vaccine and their 16 litter mates served as controls. Two of the vaccinated ones died; one death could be attributed to

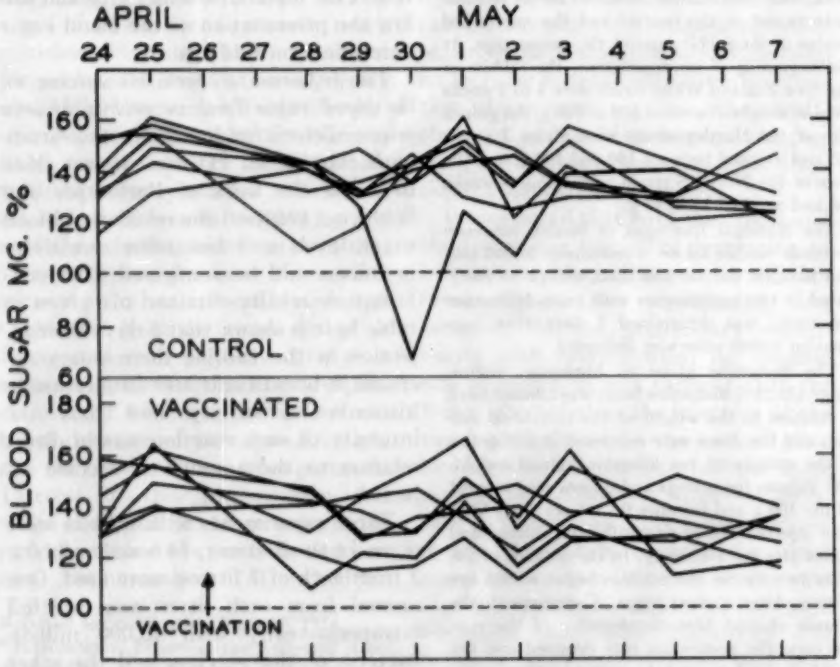

FIG. 1.—Daily determinations of the sugar in the blood of rabbits after one intraperitoneal injection of 40×10^9 bacteria of pertussis vaccine.

TABLE 2.—*Blood sugar levels and histamine sensitivity of mice after injection of toxic and detoxified* H. pertussis *bacterial suspensions.*

Time, days	Blood sugar levels*				Histamine sensitivity				
	Toxic**		Detoxified**	Controls	Number of bacteria	Histamine,	Toxic	Detoxified	Controls
	D/n†	mg %	mg %	mg %	$\times 10^9$	mg/kg	D/n	D/n	D/n
					Bacterial suspension no. 1				
					6000	50	3/3‡	14/20	
4	5/16	87.5	101.8	120.0#	1500		6/20	10/20	
5	5/16	89.5	116.8	163.6	375		3/20	1/20	
					94		1/20	0/20	
	Avg.	88.5	109.3	141.8	None				0/20
					Bacterial suspension no. 2				
					4000	100	15/20	14/20	
4	1/16	101.0	109.0	141.0	1000		13/20	7/20	
5	2/16	109.0	116.0	152.0	250		1/20	3/20	
					62		1/20	1/20	
	Avg.	105.0	112.5	146.5	None				0/20

* Each blood sugar level given represents the average value of the 3-mouse-pools determinations of 15 mice except in those groups where there were less than 15 mice due to toxic deaths. Each group contained an extra mouse for substitution if needed.
** Number of bacteria injected, 4000×10^9.
† Deaths before sugar determinations were made. There were no deaths in the detoxified vaccine or control groups.
‡ Nine mice died from toxin and 8 were unaccounted for.
# Starvation period was 10 hours instead of 6 hours.

diarrhea and the other possibly to the vaccine. None of the control rabbits died. On the 5th day the average blood sugar determination for the vaccinated rabbits was 124 mg % and for the controls it was 123 mg %. On the same day, after the blood samples had been withdrawn, the rabbits were tested for histamine sensitivity. The results, given later in table 4 (test no. 2), show that only 3 of the 14 vaccinated rabbits died, in contrast to 8 of the 16 control rabbits. The histamine sensitivity portion of the test was repeated. This time the difference in reactivity was not as great but it was in the same direction; 8 of 15 vaccinated rabbits and 10 of 16 controls died. The results of this test will also be given in table 4 (test no. 3).

In the third and final blood sugar experiment with rabbits, 12 rabbits were injected intravenously with three doses of 20,000 million bacteria each, at intervals of 3 and 4 days. Seven died and most of the other 5 lost in weight. Blood sugar determinations were begun 2 days before the first injection and were made daily until 5 days after the last injection. The results given in figure 2 show that there was a gradual drop in blood sugar and that it was most marked in those animals that died. The 5 survivors and 8 controls, 5 of which were litter mates of the 5 vaccinated survivors, were injected with histamine. None of the vaccinated rabbits showed a reaction, whereas 3 of the control litter mates died (see table 4, test no. 4). Among the other 3 controls, one died and another had servere convulsions but survived. In this test the drop in blood sugar was not correlated with an increase in histamine sensitivity.

Attention was next directed to the effect of toxic vaccines on the blood sugar and histamine sensitivity of mice. Pittman (1951b) had observed less sensitization in mice after inoculation of a toxic vaccine than after a detoxified one. Two experiments were done at different times. For each, a fresh suspension of *H. pertussis* bacteria was prepared with a portion detoxified by heating at 56 C for 30 minutes. The blood sugar determinations were made 4 and 5 days after injection and the sensitivity test after 5 days. All injections were made intraperitoneally. The results are given

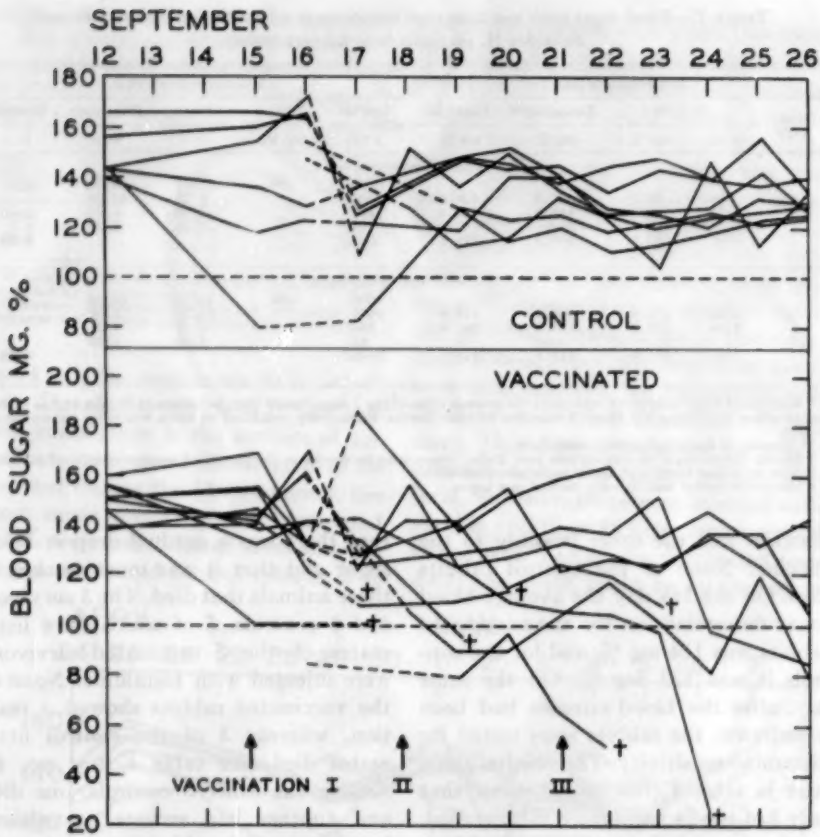

FIG. 2.—Daily determinations of the sugar in the blood of rabbits after 3 intravenous injections each of 20×10^9 bacteria of pertussis vaccine. Beginning September 17 the rabbits were starved before bleeding, for 12 hours instead of 6 hours. † indicates the death of an animal.

TABLE 3.—*Histamine sensitivity of rabbits after intraperitoneal injection of pertussis vaccine.*

Exp. number and date	Vaccine dose, number bacteria $\times 10^9$	Severe reactions–deaths/number injected				
		Histamine diphosphate dose-mg/kg				
		4.0	3.0	2.0	1.4	1.0
1a 3/10/52	none 10 40		 5–3/6 5–2/6	2–1/6 3–1/6 2–1/6	3–2/6 2–1/6 2–1/6	2–2/6
1b 11/28/52	none 40		5–5/8 5–3/8			
5 2/11/53	none 20	14–13/16 11–10/16			2–1/9 0–0/3	

There was a 5-day interval between the injection of vaccine and of histamine.

TABLE 4.—*Summary: The reduction of histamine sensitivity of rabbits following injection of pertussis vaccine.*

Histamine diphosphate, mg/kg	Serial number of sensitivity tests							Totals	
	1			2	3	4 (fig. 2)	5	D/n	Percent
	a	b	a						
Controls—not vaccinated									
4.0							13/16*	13/16	81.2
3.0		5/8		5/8	6/8			16/24	66.7
2.0	1/6			3/8	4/8	3/5		11/27	40.7
1.4	2/6						1/9	3/15	20.0
Vaccinated—number of bacteria $\times 10^9$ and route of injection									
	40,	ip	10, ip	20,	iv	20(3x)iv	20, ip		
4.0							10/16	10/16	62.5
3.0	2/6	3/8	3/6	2/7	4/7			14/34	41.2
2.0	1/6		1/6	1/7	4/8	0/5		7/32	21.9
1.4	1/6		1/6				0/3	2/15	13.3

The rabbits in no. 2 were from the second blood sugar experiment, in no. 3 they were from the repeated histamine sensitivity test of no. 2, and in no. 4 they were from the third rabbit blood sugar experiment. No. 5 test was done to provide a better distribution of the animals.
* Number of fatal shocks/number injected.

in table 2. With each suspension, both toxic and detoxified, a drop in blood sugar was induced but lower values were obtained with the mice that received the toxic than the detoxified portions. The latter difference, however, was much greater with the mice that received the respective portions of no. 1 than of no. 2. The toxic portion of no. 1 was apparently more toxic than the corresponding portion of no. 2: deaths in the respective groups of 32 each, before the sugar determinations were made were 10 and 3. The results suggest that the presence of heat-labile toxin tended to intensify the hypoglycemia in mice induced by pertussis vaccine. On the other hand, these two experiments did not show a significant difference in the histamine sensitivity of the mice injected with the toxic and detoxified portions. It therefore is suggestive that the reduction in blood sugar caused by the heat-labile toxin is not correlated with an increase in histamine sensitivity. The results, however, cannot be considered conclusive.

The influence of pertussis vaccine on the histamine sensitivity of rabbits.—In order to show both the fatal and nonfatal reactions of the rabbits to histamine, the observations of three experiments are recorded in table 3. There was a tendency for more of the controls exhibiting severe shock to die than of the vaccinated rabbits: 54.9% of the controls had severe shock and 47.0% died while 55.6% of the vaccinated ones had severe shock and 34.9% died.

In each of 5 tests it was observed that at a given dose of histamine there were usually more and at no time less of the controls to die than of the vaccinated rabbits. But the difference was not significant in a single test. When all of the tests were pooled, however, the difference, although slight, was significant. In table 4 is summarized the number of fatal shock reactions that were observed in the 5 tests and in figure 3 is given the dosage response curves for the vaccinated and control groups. The curves and the log LD_{50} for each group were determined by the Bliss-Fisher method. The lines were parallel and the differnece between the two LD_{50}'s was significant at less than 5%.*

The influence of pertussis vaccine on the histamine sensitivity of guinea pigs.—

* The calculations done by Mr. J. E. Lieberman are gratefully acknowledged.

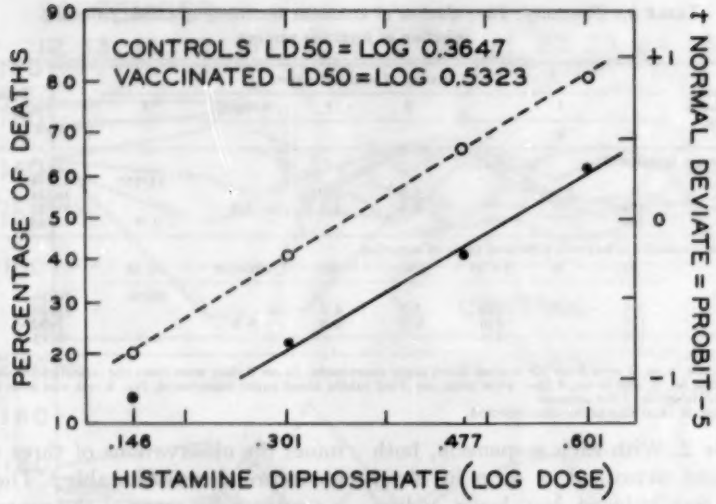

FIG. 3.—Histamine sensitivity of control and pertussis-vaccinated rabbits from table 4.

The results of an experiment are given in table 5. The vaccinated guinea pigs were injected intraperitoneally with 25,000 million bacteria of the vaccine. This dose for a 150 g guinea pig is equivalent to a dose of 2500 million, which for a 15 g mouse is sufficient to induce a 50- to 100-fold increase in histamine sensitivity. The interval between injection of the vaccine and histamine was 5 days. There were 24 vaccinated guinea pigs and 32 controls. The extra 8 controls were included to provide an additional group for determining the sensitivity of the normal animals in view of the variation in sensitivity that may exist from batch to batch of guinea pigs (Wilson, Booth and DeEds, 1950). The approximate LD_{50} had been determined in a preliminary test that suggested a decrease in sensitivity in vaccinated guinea pigs. The doses of histamine diphosphate were varied by a 1.23-fold increment. Litter mates were paired as far as possible in the vaccinated and control groups that received the same dose of histamine.

TABLE 5.—*Reduction of histamine sensitivity of guinea pigs following intraperitoneal injection of pertussis vaccine.*

Vaccine dose, number bacteria $\times 10^6$	Histamine diphosphate-mg/kg			Totals	
	1.2	0.98	0.8	D/n	Percent
None	7/8*	4/8	3/8	14/24	58.3
25	2/8	1/8	2/8	5/24	20.8

An additional group of 8 control guinea pigs were injected with 0.65 mg/kg of histamine diphosphate; these was one fatal shock.
* Number of fatal shocks/number injected.

Deaths in the four control groups ranged from 12.5 to 87.5%. The LD_{50} was near 0.9 mg/kg. In contrast, no more than 25% of the guinea pigs in any one of the three groups of vaccinated animals died, and it was not practical to calculate the LD_{50} to compare with the LD_{50} for the controls. However, when the vaccinated animals that received the three doses of histamine were pooled and compared with a pool of the correspondingly treated controls, it was found that there was a significant difference between the response of the two groups. The probability that the difference is due to chance is less than 5%.

Since the LD_{50} of histamine for the vaccinated guinea pigs was not determined, the relative reduction induced in the guinea pig and the rabbit is not known. It is suggestive, however, that the decrease in the guinea pig was of a greater degree than in the rabbit. The guinea pig is naturally more sensitive than the rabbit. The LD_{50} of histamine for each, observed in this work, was 0.9 and 2.3 mg/kg, respectively.

DISCUSSION

In contrast to the 50- to 100-fold increase in histamine sensitivity induced by pertussis vaccine in the mouse, it was observed that this vaccine induced a slight but definite decrease in histamine sensitivity in two animals, the rabbit and guinea pig, that are normally much more susceptible to histamine than the mouse. The decrease in the guinea pig, normally more sensitive than the rabbit, appeared to be of a slightly greater degree than in the rabbit. The graded doses of histamine used were not large enough to obtain a graded response in the vaccinated guinea pigs. In rabbits, however, the graded decrease in response obtained was parallel to the response of normal rabbits. The degree of decrease was similar to the decrease in susceptibility to anaphylactic shock observed by Hoene et al (1952) in horse serum sensitized guinea pigs treated with ACTH before injection of graded doses of antigen.

In the vaccinated mouse, susceptibility to histamine is in the same order of magnitude as after adrenalectomy (Halpern and Wood, 1950a). Because of this similarity there has been some speculation on the role of the adrenal in the vaccine induced sensitivity. Both the vaccinated and the adrenalectomized mouse react in like manner to certain agents but different to others. For example, the adrenalectomized mouse can tolerate (1) 50 to 100 lethal doses of histamine when treated with the antihistaminic, phenergan (Halpern and Wood, 1950a,b), (2) 5 lethal doses when treated with adrenalin (Halpern and Wood, 1950b) and (3) 5 to 10 lethal doses when treated with cortisone but 50 to 100 lethal doses when treated with both adrenalin and cortisone (Halpern, Benacerraf and Briot, 1952). The pertussis-vaccinated mouse can likewise tolerate histamine, the same as the normal mouse, when treated with phenergan (Halpern and Roux, 1949 and Pittman, 1953); adrenalin, however, does not increase its tolerance (Halpern and Benacerraf, 1950) and the protective effect of cortisone seems to be of a lower degree—reported to be against about one LD_{50} of histamine (Kind and Parfentjev, 1951; Kind, 1953; Malkiel and Hargis, 1952b). In addition, the vaccinated mouse (Kind and Gadsen, 1953), like the adrenalectomized rat (Thorn et al, 1942), has a decreased tolerance to reduced oxygen tension but, unlike the rat, treatment with cortical extract does not restore tolerance. With the hope of throwing some light on the possible role of the adrenal in the pertussis-vaccinated mouse some work by the authors is in progress.

Besides the contrasting difference in histamine sensitivity induced by pertussis vaccine in the rabbit and mouse, one other difference was noted. It is that in the former there was no change in blood sugar unless the amount of vaccine approached the lethal dose, whereas in the mouse there was a decrease which paralleled the increase in histamine sensitivity. With toxic vaccines, however, a further decrease in blood sugar in the mouse was not accompanied by a further increase in histamine sensitivity. It is suggested that when the labile toxin plays an important role in the decrease in blood sugar, there is no interrelation

with histamine sensitivity in the mouse. The question arises as to whether or not the drop in blood sugar following injection of detoxified vaccine is due to another reactive substance unrelated to the histamine sensitizing property.

Oddy and Evans (1940) and Evans (1943) showed that a detoxified extract of *H. pertussis*, injected intravenously in rabbits, induced a temporary hyperglycemia which returned to normal in about 8 hours, while a heat-labile toxic extract, after a temporary hyperglycemia, induced a hypoglycemia that progressed until death, which occurred within 24 hours. In the present work, the lack of change in blood sugar 1 day after the injection of a vaccine (devoid of heat-labile toxin) is in keeping with these authors' 24-hour observations made with a detoxified extract; it was only after repeated doses of the vaccine that a lowering of blood sugar and death occurred.

The fact that animals of two species normally sensitive to histamine were not rendered more sensitive by pertussis vaccine but, on the contrary, became slightly more resistant suggests that a general rise in histamine sensitivity would not be expected in the child suffering from pertussis. In a personal communication from Dr. Manfred Weichsel of Sunnyside, New York, it was reported that strongly positive reactions to histamine in children suffering from pertussis were obtained but similar reactions were also obtained in the controls. No definite conclusions could be drawn.

SUMMARY

1. In contrast to the many fold increase in histamine susceptibility induced by pertussis vaccine in mice, both rabbits and guinea pigs showed a slight but significant decrease in histamine sensitivity after injection of the vaccine.

2. In an attempt to use a decrease in blood sugar as an indication of increased sensitivity, as had been shown, and confirmed in this paper, to parallel the histamine reaction in the mouse, it was found that there was no decrease in blood sugar in the rabbit except when a near fatal dose of vaccine was given.

3. A toxic vaccine caused a greater drop in blood sugar in the mouse than a detoxified vaccine. This reaction was not correlated with an increase in histamine sensitivity.

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THE COMPARATIVE EFFECTIVENESS OF AUREOMYCIN, TERRAMYCIN, CHLORAMPHENICOL, ERYTHROMYCIN, AND THIOCYMETIN IN SUPPRESSING EXPERIMENTAL RICKETTSIAL INFECTIONS IN CHICK EMBRYOS

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Aureomycin, terramycin, and chloramphenicol have been shown to be effective in the treatment of various experimental rickettsial infections in chick embryos.[1–9] Erythromycin[10,11] and thiocymetin[12,13] are broad-spectrum antibiotics active against a wide variety of both Gram-positive and Gram-negative bacteria. Their therapeutive effectiveness against rickettsial infections has not been previously reported.

The purpose of this paper is to present data demonstrating the comparative effectiveness of these five compounds in suppressing experimental infections caused by *Rickettsia rickettsii*, *R. akari*, *R. prowazekii*, *R. typhi*, and *Coxiella burnetii*. The data dealing with aureomycin, terramycin, and chloramphenicol corroborate the work of previous investigators, particularly that of Jackson,[9] with which it can be most closely compared. The results with these three compounds furnish a basis for the

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comparison of the corresponding data obtained with erythromycin and thiocymetin.

MATERIALS AND METHODS

The following substances were tested: aureomycin HCl (mol. wt. 520), terramycin HCl (mol. wt. 497), chloramphenicol (mol. wt. 323), erythromycin (mol. wt. ca. 725), and thiocymetin (mol. wt. 356). The structure of erythromycin is unknown although the approximate empirical formula of $C_{34-36}H_{60-62}NO_{11-14}$ has been reported.[10] Thiocymetin is a commercial name for Dd-threo-2-dichloroacetamido-1-(4-methylsulfonylphenyl)-1,3-propanediol. It is a synthetic compound, closely related to chloramphenicol in structure.

The following organisms were used in the tests: *R. rickettsii* (Sheila Smith strain), *R. prowazekii* (Breinl strain), *R. typhi* (Wilmington strain), *R. akari* (Davis #7 strain) and *C. burnetii* (Nine-Mile, California, Adohr, and Idaho strains). The data on *C. burnetii* were obtained with the Nine-Mile strain unless otherwise noted.

Standard infectious inoculums were assured by using aliquots of single pools of infected yolk-sac suspensions (10%, w/v) stored at −62 C in a solid CO_2 refrigerator until used.

The test employed groups of 20 to 50 six-day-old chick embryos which were injected in the yolk sac with 0.2 ml aliquots of the appropriate infectious yolk-sac suspension diluted to a concentration such that the mean death time of untreated groups lay between 5 and 7 days post-injection. Twenty-four hours later the embryos were injected by the same route with 0.5 ml aliquots of an aqueous solution of drug in an amount previously determined to be below a toxic level for chick embryos. Controls were injected with 0.5 ml aliquots of 0.15 *M* NaCl. The embryos were then held at 35 C, candled daily, and deaths recorded. Those embryos dying within 72 hours after injection of the infectious suspension (15% on the average) were discarded and not counted, on the well-based assumption that such deaths are due to the trauma of injection. The tests were terminated on the 14 day after injection of the infectious suspension. For purposes of calculation all embryos still alive on this day were assumed to be dead. The difference in mean death time between the treated groups and the control groups was taken as the measure of effect of the compound under test.

RESULTS

The results are summarized in table 1. In most cases the data for any given concentration of drug represent the pooled data from several experiments. This is particularly so with the control embryos, since in these cases it was often possible to use embryos which were actually being used in connection with unrelated experiments as control material.

An estimate of the statistical reliability of the data in table 1 was made by calculating the standard errors (standard deviation/$\sqrt{n}$) of the mean death times. Including all treated groups, the standard error ranged from ±0.01 to ±0.80 days. The higher the dose of drug given, the more irregular was the death curve, and consequently the larger was the error. This was true in all cases except that of chick embryos infected with *R. typhi* and receiving 0.1 mg of thiocymetin. In this case only 2 out of 53 embryos had died (table 1) by the time the experiment was terminated. Since the remaining 51 embryos were dead by definition on the 14th day postinfection, the standard error was naturally very small, i.e., ±0.01 days. A better idea of the range of standard errors involved can be given by listing the range found in all the tests for each organism concerned. These are as follows: *R. rickettsii*, ±0.13 to 0.47 days; *R. akari*, ±0.11 to 0.62 days; *R. prowazekii*, ±0.09 to 0.73 days, *R. typhi*, ±0.01 to 0.51 days; *C. burnetii*, ±0.15 to 0.80 days. The range for *C. burnetii* when the negative tests with thiocymetin are excluded is ±0.15 to 0.44 days. The average of all standard errors in the treated groups is ±0.26 days. In the untreated but infected controls the standard errors are as follows: *R. rickettsii*, ±0.05 days, *R. akari*, ±0.05 days; *R. prowazekii*, ±0.04 days; *R. typhi*, ±0.14 days; *C. burnetii*, ±0.05 days.

The data presented in table 1 indicate striking differences among the organisms tested with respect to their sus-

TABLE 1.—*The effect of aureomycin, terramycin, erythromycin, chloramphnicol, and thiocymetin on experimental infections of* R. rickettsii, R. akari, R. prowazekii, R. typhi, *and* C. burnetii *in chick embryos.*

Organism	Aureomycin				Terramycin				Erythromycin				Chloramphenicol				Thiocymetin			
	Mg per embryo	Number of embryos	MD. exp.	Protection	Mg per embryo	Number of embryos	MD. exp.	Protection	Mg per embryo	Number of embryos	MD. exp.	Protection	Mg per embryo	Number of embryos	MD. exp.	Protection	Mg per embryo	Number of embryos	MD. exp.	Protection
R. rickettsii	0.250	48/50	10.7+	5.7+	0.025	69/69	10.8	5.8	0.230	49/50	8.9+	3.9+	0.323	42/42	10.0	5.0	0.021	24/24	10.2	5.2
Number of	0.125	48/48	9.1	4.1	0.019	73/73	10.3	5.3	0.180	57/57	8.1	3.1	0.242	43/43	8.9	3.9	0.014	92/92	9.1	4.1
embryos =195	0.064	51/51	7.3	2.3	0.012	75/75	8.2	3.2	0.130	52/52	7.2	2.2	0.162	45/45	8.1	3.1	0.011	48/48	7.8	2.8
Control	0.032	53/53	6.3	1.3	0.006	45/45	6.7	1.7	0.080	54/54	6.2	1.2	0.081	43/43	6.4	1.4	0.007	67/67	6.3	1.3
MD =5.0																	0.004	69/69	5.6	0.6
R. akari	0.130	73/73	10.1	4.0	0.020	19/20	10.0+	3.9+	0.290	23/23	8.5	2.4	0.242	75/75	10.4	4.3	0.018	40/41	10.6+	4.5+
Number of	0.064	22/22	7.7	1.6	0.015	23/23	9.3	3.2	0.180	29/29	8.4	2.3	0.162	23/23	9.3	3.2	0.014	40/42	9.2+	3.1+
embryos =210	0.032	46/46	7.2	1.1	0.010	24/24	8.0	1.9	0.080	25/25	8.0	1.9	0.081	50/50	7.7	1.6	0.009	49/49	8.1	2.0
Control	0.016	45/45	6.5	0.4	0.005	22/22	7.0	0.9	0.043	19/19	6.4	0.3	0.041	50/50	6.8	0.7	0.005	54/54	6.8	0.7
MD =6.1																				
R. prowazeki	0.258	22/51	12.6+	6.8+	0.025	15/21	10.5+	4.7+	0.029	37/48	10.4+	4.6+	0.323	39/43	10.9+	5.1+	0.018	27/31	9.1+	3.3+
Number of	0.194	42/42	11.6	5.8	0.020	22/29	9.9+	4.1+	0.015	39/41	8.8+	3.0+	0.242	50/50	9.6	3.8	0.014	35/37	8.5+	2.7+
embryos =210	0.129	76/85	10.6+	4.8+	0.015	31/34	9.3+	3.5+	0.007	47/47	7.1	1.3	0.162	48/48	8.4	2.6	0.011	31/31	6.8	1.0
Control	0.065	79/80	7.7+	1.9+	0.010	34/34	8.0	2.2	0.004	49/49	6.4	0.6	0.081	43/43	7.2	1.4	0.009	42/42	6.7	0.9
MD =5.8	0.032	79/79	6.7	0.9	0.005	53/53	6.4	0.6									0.007	49/49	6.2	0.4
C. burnetii	1.00	19/74	13.3+	6.5+	0.063	34/72	12.4+	5.6+	0.700	28/28	8.1	1.3	1.00	71/71	9.7	2.9	0.10	27/27	7.2	0.4
Number of	0.50	38/73	13.2+	6.4+	0.031	48/73	12.2+	5.4+	0.350	25/25	7.5	0.7	0.500	74/74	9.2	2.4	0.03	15/16	7.1+	0.3
embryos =747	0.25	66/78	12.2+	5.4+	0.015	112/112	10.2	3.4	0.080	27/27	6.9	0.1	0.250	75/75	8.3	1.5				
Control	0.125	77/77	10.1	3.3	0.008	93/93	8.9	2.1					0.125	73/73	7.2	0.4				
MD =6.8	0.063	77/77	8.4	1.6																
R. typhi	0.25	2/54	13.6+	6.9+					0.08	9/26	13.5+	6.8+					0.10	2/53	13.8+	7.1+
Number of	0.125	74/76	11.8+	5.1+					0.04	17/24	13.5+	6.8+								
embryos =99																				
Control																				
MD =6.7																				

MD. =mean death; Protection =difference between MD of controls and MD of treated groups (MD. exp.). In columns headed "Number of embryos" the first figure is the number that actually died within the period of the test; the second figure is the total number of embryos involved in test.

The plus sign following several of the data indicates that the value is actually a minimum value, because some of the treated embryos did not die within the experimental period of 14 days following infection.

TABLE 2.—*The concentration of drug causing a delay of 4 days in mean death time of experimentally infected chick embryos.*

Drug	Aureomycin			Terramycin			Chloramphenicol			Erythromycin			Thiocymetin		
Organism	μM/kg	mg/kg	$\frac{1}{\text{slope}}$	μM/kg	mg/kg	$\frac{1}{\text{slope}}$	μM/kg	mg/kg	$\frac{1}{\text{slope}}$	μM/kg	mg/kg	$\frac{1}{\text{slope}}$	μM/kg	mg/kg	$\frac{1}{\text{slope}}$
R. rickettsii	4.17	2.12	4.9	0.47	0.23	7.8	11.8	3.83	5.7	5.59	4.05	4.1	0.67	0.23	7.6
R. akari	4.14	2.14	8.0	0.63	0.32	5.7	10.6	3.44	4.9	12.3	8.95	3.3	0.75	0.27	17.0
R. prowazeki	3.74	1.93	5.7	0.60	0.30	6.0	13.9	4.48	5.1	0.53	0.38	13.9	0.85	0.30	4.5
C. burnetii	4.70	2.54	6.9	0.53	0.27	6.9	98.2	31.7	3.0						

$\frac{1}{\text{slope}}$ =days of delay in mean death time per 10-fold increase in drug dose.

The columns "μM/kg" and "mg/kg" are based on an average egg weight of 60 g.

ceptibility to the different compounds. *R. rickettsii* and *R. akari* are highly sensitive to thiocymetin and terramycin, less sensitive to aureomycin, chloramphenicol, and erythromycin. *R. prowazekii* and *R. typhi* are most sensitive to erythromycin and terramycin, less sensitive to thiocymetin and aureomycin, and least sensitive to chloramphenicol. *C. burnetii* is most susceptible to the action of terramycin, somewhat less so to aureomycin, relatively insensitive to high concentrations of chloramphenicol, and essentially unaffected by maximum tolerated doses of thiocymetin or erythromycin. Similar results with respect to thiocymetin were obtained also with California, Adohr, and Idaho strains of *C. burnetii* (not shown in table 1).

Results which were in general agreement with those given in table 1 were obtained by Jackson[9] with aureomycin, terramycin, and chloramphenicol. She also noted that *C. burnetii* was most susceptible to the action of terramycin and least sensitive to chloramphenicol.

These relationships are brought out more clearly by the data given in table 2. Here the concentrations of the various compounds causing a delay of 4 days in the mean death time have been selected arbitrarily for comparison. The data in table 1 have been graphed as log of dosage in μM/response plots and the best straight line by inspection drawn through the points. The reciprocal of the slope of such curves (the column headed "1/slope" in table 2) represents delay in mean death time per log unit of drug and is another means of estimating the relative effectiveness of these compounds, at least with this test system and over the concentration range which it was possible to study. Thus table 2 indicates the relative effectiveness of the various compounds with respect to absolute concentrations (the columns headed μM/kg or mg/kg) required to produce a constant degree of protection (in this case a 4-day period of protection was arbitrarily selected) and also with respect to the rate of increase in protection with increasing drug concentration. Such calculations for *R. typhi* are not included in table 2 because of the lack of data covering sufficiently wide concentration ranges.

The relative effectiveness of the drugs tested against any given organism varied somewhat, depending upon which criterion was applied. Thus in terms of absolute concentration, *R. rickettsii* was more sensitive to aureomycin than to chloramphenicol, since it required 4.17 μM/kg and 11.8 μM/kg, respectively, to cause a delay of 4 days in the mean death time (table 2). On the other hand, when the number of days delay in mean death time per log unit of drug is used in comparing these two compounds (columns headed 1/slope in table 2), it is apparent that *R. rickettsii* is more responsive to an increase in the dose of chloramphenicol than to an equivalent increase in the amount of aureomycin administered. The organisms employed in these studies were ranked by taking both these methods of estimating sensitivity into account.

The columns headed "mg/kg" in table 2 were included for convenience of those readers accustomed to think in terms of weights of drug rather than in terms of molar quantities. The more illuminating method of examining such data is that based on molar quantities.

DISCUSSION

The results of these tests indicate that different species of pathogenic rickettsiae vary significantly in their susceptibility to the action of the antibiotics and chemotherapeutic agents assayed. This conclusion, of course, is similar to the results with pathogenic

bacteria obtained by many other investigators.

Such differences as appear in the data in tables 1 and 2 are largely qualitative differences, with the exception of results obtained with *C. burnetii* treated with thiocymetin. In this case maximum tolerated doses of thiocymetin (0.10 mg/chick embryo) produced no significant delay in the mean death time of infected embryos. This is a great contrast with the corresponding tests involving *R. rickettsii*, in which 0.021 mg/chick embryo of thiocymetin causes a delay of 5.2 days in the mean death time of infected embryos.

The results obtained with thiocymetin are of interest in comparison with those resulting from the chloramphenicol tests. The two compounds are closely related chemically, the only difference between the two being the presence in thiocymetin of a methylsulfonyl group on the phenyl ring in place of the similarly situated nitro group in chloramphenicol. The introduction of the methylsulfonyl group in place of the nitro group decreased the maximum tolerated dose (MTD) in chick embryos by a factor of about 10 (MTD = 0.1 mg/embryo). The effect on *R. rickettsii*, *R. akari*, and *R. prowazekii* was to enhance the suppressive effect of the compound by a factor of about 16. The effect on *C. burnetii* was negligible, as can be seen in table 1; 0.10 mg/embryo of thiocymetin produced the statistically insignificant delay in mean death time of 0.4 days, while a dose of 0.125 mg/embryo of chloramphenicol produced likewise the insignificant delay of 0.4 days.

The data in table 2 indicate that aureomycin and terramycin were the most consistent in their effect against the entire group of organisms tested. This was true with respect both to the amount necessary to produce a given protective effect and to the slopes of the dosage-response curves that can be constructed from the data in table 1. This might be interpreted as indicating that these closely related compounds affect fundamental metabolic systems common to all the species of rickettsiae examined. Chloramphenicol appeared to be much less effective against *C. burnetii* than against the other three organisms, which were affected about equally. Erythromycin, which gave a minimal delay in death in embryos infected with *C. burnetii*, produced a significantly greater effect against *R. prowazekii* than against *R. rickettsii* or *R. akari*. Thiocymetin, although it was equally effective against the latter three rickettsiae with respect to the amount necessary to cause a 4-day delay in mean death time, gave a dosage-response curve with a slope considerably greater when tested against *R. akari* as compared to *R. rickettsii* or *R. prowazekii*.

C. burnetii was clearly distinguished from the other rickettsiae tested by virtue of its relative insensitivity to erythromycin, chloramphenicol, and thiocymetin. This result adds to the body of evidence which tends to differentiate this organism from the rest of the group of pathogenic rickettsiae and which has resulted in its being placed in the genus *Coxiella*.

Some of the characteristics of the dosage-response curves which may be constructed from the data in table 1 should be mentioned. In some cases the points are scattered in such a fashion that it is difficult to judge by inspection just where the curve lies. This means that the figures in table 2 which are derived from the original data given in table 1 are only approximate figures.

In some cases, notably that of *R. rickettsii* versus erythromycin, an arithmetic plot of μM versus days delay in mean death time gives a straight line which appears to fit the data better than does a straight line drawn through the points of the usual log of concentra-

tion versus response plot of the data. This is probably due to the narrow drug concentration range within which it was necessary to work rather than to a different type of response.

When the logarithm of drug concentration was plotted against the days delay in mean death time, the data appeared to give two curves with quite different slopes in several cases. This phenomenon can be seen if the data in table 1 involving *R. akari* versus aureomycin or thiocymetin, and *R. rickettsii* versus erythromycin are so graphed. In these cases the first three or four points give one curve and the points involving higher concentrations give another curve. In such cases, the data in the column "1/slope" in table 2 have been based on the higher concentrations and therefore on the second curve, which in every case has a smaller slope than the curve covering the lower drug concentrations. The reasons for this type of response are not immediately apparent. It is quite possible that the antibiotic in these cases inhibits one metabolic system in the lower concentration range and another system in the higher concentration range, and that the combining ratios of drug to enzyme (assuming that the action is due to enzyme inhibition) are sufficiently different from each other to produce curves with significantly different slopes when plotted as described above. Such a situation has been found to exist in yeast,[14] free-living protozoa,[15] and sea urchin eggs.[16] In the case of the free-living protozoan it was shown that cell division was inhibited only within the higher concentration range of ethyl carbamate, although inhibition of a major portion of the over-all oxygen consumption of the ciliates was apparent within the lower concentration range. In the present case it is unfortunately not possible to analyze the findings in a similar manner.

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SUMMARY

1. Comparative tests of aureomycin, terramycin, chloramphenicol, erythromycin, and thiocymetin indicated that aureomycin and terramycin possessed the highest and most consistent death-delaying activity in chick embryos infected with *Rickettsia rickettsii*, *R. akari*, *R. prowazekii*, *R. typhi*, or *Coxiella burnetii*, and that terramycin was the most effective.

2. Thiocymetin was equivalent to terramycin in its effect against *R. rickettsii*, *R. prowazekii*, and probably *R. typhi*, but was without effect on *C. burnetii* at maximum tolerated doses. Its effect against *R. akari* was greater than that of terramycin.

3. Erythromycin showed outstanding activity against *R. prowazekii*, activity greater than aureomycin against *R. typhi*, moderate activity against *R. rickettsii* and *R. akari*, and a small effect against *C. burnetii* when given in maximum tolerated doses.

4. Chloramphenicol demonstrated moderate activity against *R. rickettsii*, *R. akari*, and *R. prowazekii*, but only small activity against *C. burnetii* even when employed at very high doses.

5. The characteristics of the responses of the various rickettsiae to the different compounds are described.

STUDIES ON IMMUNIZATION AGAINST PLAGUE

IX. THE EFFECT OF CORTISONE ON MOUSE RESISTANCE TO ATTENUATED STRAINS OF *Pasteurella pestis**

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Interest in the development of effective vaccines against plague has led to the isolation and study of *Pasteurella pestis* strains of lowered virulence and to fairly extensive use of living, so-called "avirulent" strains in vaccines for human populations.[1–5] Other than their inability to produce progressive fatal infection in a susceptible host, these attenuated strains do not uniformly possess characteristics that distinguish them from fully virulent strains. Some of these strains, including ones extensively used as living vaccines, can actually multiply appreciably in mice and guinea pigs, although this multiplication is controlled before it assumes serious proportions.[6] Although under ordinary circumstances these strains are relatively harmless, even when inoculated in very large numbers, evidence is here presented that certain of these strains, under conditions of altered host physiology, are capable of producing in mice widespread invasive infection ending in bacteremia and death.

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MATERIALS AND METHODS

Mice.—Albino mice weighing 18 to 20 g were used in all experiments; in most cases, and unless otherwise noted, they were of the Princeton strain.

Cortisone.—Cortisone (Cortone Acetate, Merck & Co.) was diluted in 0.85% sodium chloride to yield the desired dose in 0.1 ml. In all cases this was injected in a single dose intramuscularly into the hind legs of mice, and was given 4 hours prior to injection of bacteria or toxin.

Infecting organisms.—The strains of *Past. pestis* of low virulence—E.V. 76, A1122, Tjiwidej and F7793–10—previously described and discussed in detail,[6] were grown on blood agar at 37 C for 48 hours and harvested in saline. Dilutions were made in saline to yield the appropriate number of organisms in 0.5 ml.

Toxin.—The endotoxin of *Past. pestis* was prepared according to the method of Englesberg and Levy.[7] Strain E.V. 76 was cultured at 30 C for 8 days in a casein hydrolysate mineral glucose medium. The supernatant fluid was removed and brought to saturation with ammonium sulfate by the addition of dry reagent. The precipitate which formed was collected and stored in the dry state. At the time of use the dried protein was redissolved in *M*/60 phosphate-buffered saline at pH 7.0. This toxin contained 15.7% fraction I by weight.

Envelope antigen.—Fraction I, the water-soluble envelope antigen, was extracted from acetone-killed dried *Past. pestis* (strain Yreka), precipitated and crystallized according to the method of Baker and his associates.[8]

Hyperimmune globulin.—A commercial prepa-

7. Englesberg, E. and Levy, J. B. 1954, J. Bact. **68**: 57–60.
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ration of hyperimmune globulin—anti-plague immune serum globulin (rabbit), Lederle—was employed for passive immunization. The lyophilized globulin was dissolved in saline to yield 0.3 MPU* in 0.5 ml.

Methods of immunisation.—The toxin was administered subcutaneously, 0.25 LD_{50} of 0.2 ml in saline every other day for two 4-dose courses 8 days apart. When fraction I was used, a 0.1% solution was given intraperitoneally in a total dose of 0.8 mg per mouse. This was divided into four equal doses and was administered at weekly intervals. The antiplague globulin was given at the time of challenge, 0.3 MPU in 0.5 ml of saline intravenously.

Methods of detection of infecting organisms.—Using aseptic technique, 2 or 3 drops of heart blood or the cut surface of spleen was placed on blood agar plates, streaked out and incubated for 48 to 72 hours at 37 C. *Past. pestis* was identified primarily by colony characteristics and cell morphology. In addition to cultures, microscopic sections and stains of various tissues were made.

EXPERIMENTS AND RESULTS

The effect of cortisone on mouse resistance to strain E.V. 76.—This strain, extensively used in a living vaccine for human populations,[2,3] possesses some capacity for multiplication in tissues of normal mice, but this multiplication is limited, localized and suppressed without widespread invasion or bacteremia.[6] The effect of cortisone on mouse resistance to this strain was studied using graduated doses of organisms (20,000 to 500,000 viable bacteria, intraperitoneally) and a fixed dose of cortisone (2.5 mg/mouse). Results of a typical experiment are presented in table 1. In this experiment mice of the Swiss-Bag strain were used. All animals inoculated with E.V. 76 but not treated with cortisone appeared healthy throughout the experiment, with the exception of a single animal in the group challenged with 500,000 organisms, and even in this mouse, autopsy and culture failed to reveal evidence that death was due to the inoculated organisms. Almost all animals treated with cortisone and challenged with strain E.V. 76 died. Even the smallest dose used, 20,000 viable bacilli, caused death of 18 of 20 mice.

TABLE 1.—*The effect of cortisone on survival of mice inoculated intraperitoneally with various doses of* Past. pestis, *strain E.V. 76.*

Viable E.V. 76 in inoculum	Survival†		
	Cortisone alone*	E.V. 76 alone	E.V. 76 with cortisone*
0	18/20		
20,000		20/20	2/20
100,000		20/20	1/20
500,000		19/20	2/20

* Dose of cortisone—2.5 mg/mouse, 4 hours prior to inoculation.
† Survivors/total inoculated.

Graduated doses of cortisone (0.05 to 2.5 mg/mouse) and a fixed dose of strain E.V. 76 were then given; the results of two such experiments are presented in table 2. Again in groups treated with cortisone and inoculated with E.V. 76 the mortality rate was markedly higher than in the untreated group and depended on the dose of cortisone used. Since Swiss-Bag mice were used in the first experiment and Princeton mice in the second, this effect of cortisone on mouse resistance is not limited to a single strain of mice.

TABLE 2.—*The effect of various doses of cortisone on survival of mice inoculated intraperitoneally with* Past. pestis, *strain E.V. 76.*

Viable E.V. 76 in inoculum	Dose of cortisone, mg*	Survival†		
		E.V. 76 alone	Cortisone alone	E.V. 76 with cortisone*
		Experiment 1		
	0	17/20		
	0.05		20/20	38/40
10,500	0.5		18/20	28/39
	1.0		19/20	13/39
	2.0		19/20	12/40
		Experiment 2		
	0	18/20		
6,400	2.0		20/20	1/20
	2.5		19/20	2/20

* Dose of cortisone—mg/mouse, 4 hours prior to inoculation.
† Survivors/total inoculated.

* One mouse protection unit (MPU) is that amount of globulin that, when administered at the time of challenge, protects 50% of the mice against 100 MLD of virulent organisms administered subcutaneously.

In these experiments the first deaths occurred on the 3rd or 4th day after inoculation, the majority from the 4th to 8th day, and a few thereafter. Cultures of heart blood collected at autopsy revealed heavy growth of *Past. pestis* in 67 to 70% of the cortisone-treated mice. In 2 to 15% of cortisone-treated mice there was no growth in cultures of blood, and in 13 to 30% the cultures contained organisms other than *Past. pestis* which possibly obscured its presence. A few mice inoculated with E.V. 76 but not treated with cortisone died, but without cultural or autopsy evidence of extensive infection or blood stream invasion.

The effect of cortisone on other strains of low virulence.—It was considered important to find out whether host resistance to strains of low virulence other than E.V. 76 would be similarly detrimentally affected by cortisone. Strains A1122, Tjiwidej and F7793-10 are ordinarily of low virulence for mice,[6] and experiments were carried out to learn the influence of cortisone on these strains.

Cortisone (2.0 mg/mouse) was given 4 hours prior to inoculation with these strains. The organisms were given intraperitoneally in the doses indicated in table 3.

Only with strain F7793-10 was the mortality rate of mice treated with cortisone markedly increased. Strains A1122 and Tjiwidej have in previous investigations[6] shown little capacity for survival and multiplication in normal mice and apparently in mice treated with cortisone are unable to produce an infection of extent sufficient to cause death.

TABLE 3.—*The effect of cortisone on survival of mice inoculated intraperitoneally with various strains of* Past. pestis.

Strain	Viable organisms in inoculum	Survival†	
		With cortisone*	Without cortisone
A1122	80,000	19/20	20/20
Tjiwidej	73,000	20/20	19/20
F7793-10	28,000	2/20	20/20
Controls	0	19/20	20/20

* Dose of cortisone—2 mg/mouse, 4 hours prior to inoculation.
† Survivors/total inoculated.

The effect of cortisone on the susceptibility of mice to the toxin of Past. pestis.—It is believed that death caused by virulent strains of *Past. pestis* is due to the release of endotoxin from lysed bacterial cells.[9] The possibility of increased susceptibility to the toxin in mice infected with an avirulent strain and treated with cortisone was investigated.

Groups of mice were pretreated with cortisone or saline and 4 hours later were inoculated intravenously with graduated doses of toxin. The results of two toxin titrations are presented in table 4.

The cortisone did not increase, but perhaps slightly decreased, the susceptibility to the toxin.

The effect of cortisone on the resistance of immunized mice to E.V. 76.—To learn whether the effect of cortisone could be neutralized by active or passive immunization, groups of mice were actively immunized with envelope antigen (fraction I) or toxin, and an additional group was passively immunized with hyperimmune globulin. The outcome of infection with E.V. 76 with and without pretreatment with cortisone was observed, and the data are presented in table 5. All of these methods of immunization clearly afforded protection.

The effect of cortisone on the gross and microscopic pathology in mice infected with attenuated strains.—The autopsy findings in mice that succumbed to infections with E.V. 76 or F7793-10 under the influence of cortisone are significant and may assist in the explanation of the events that took place:

9. Meyer, K. F. 1950, J. Immunol. **64**: 139–163.

TABLE 4.—*The effect of cortisone on survival of mice inoculated intravenously with* Past. pestis *toxin.*

	Survival* Dilution†							
	1:50	1:100	1:150	1:200	1:250	1:300	1:350	1:400	50% end point
Toxin	1/10	0/10	0/10	0/10	1/10	0/10	2/10	3/10	1:400
Toxin with cortisone‡	0/10	1/10	5/10	10/10	8/10	9/10	9/10	8/10	1:165

	Survival Dilution				
	1:10	1:40	1:160	1:640	1:2560	50% end point
Toxin	0/8	0/8	1/8	8/8	8/8	1:280
Toxin with cortisone	0/8	0/8	5/8	8/8	8/8	1:130

* Survivors/total inoculated.
† Original concentration of toxin: 1 mg/ml.
‡ Dose of cortisone—2.5 mg/mouse, 4 hours prior to inoculation.

The subcutaneous tissues and abdominal cavity were dry. The tissue at the site where the cortisone was injected seemed unchanged and was equally dry. The lungs were in every case hyperemic, quite voluminous and edematous. The auricles and the right ventricle of the heart were distended with well-clotted blood.

Most striking were the changes in the spleen, liver and intestines. The spleen was rarely enlarged, but was invariably studded with small, grayish, slightly depressed circular nodules varying in size from pinpoints to 3 or 4 mm in diameter. When death occurred 6 or 7 days after inoculation the spleen was a light gray, rather tough organ with a few minute dark red areas of spleen parenchyma. Microscopically the structure of the spleen of mice that died on the 3rd day was largely replaced by confluent masses of necrotic tissue confined to the meshes of the reticulum. The malpighian bodies appeared unchanged. Outer zones of the necrotic detritus were invaded by clusters of pale-staining bipolar bacilli intermingled with leucocytes and erythrocytes. The splenic pulp of mice that died on the 5th to 8th day was necrotic and heavily infiltrated by polymorphonuclear leucocytes, macrophages and some fibroblastic elements. In these specimens, unlike those from animals dying on the 3rd day, bacilli were not seen.

The liver was enlarged (weighing 2.3 to 2.5 g), mottled, dark brownish, hyperemic, soft, friable and studded with fine necrotic spots. In sections of tissue from animals dying on the 3rd or 4th day masses of plague bacilli were prominent in the necrotic nodules and even in the hepatic veins. Like the lesions of the spleen, those of the liver resembled granulomas by the 6th day and contained no visible bacilli.

The kidneys were enlarged, moist,

TABLE 5.—*The effect of cortisone on survival of immunized mice inoculated subcutaneously with* Past. pestis, *strain E.V. 76.*

	Survival†							
	E.V. 76 (20,000 viable organisms)				No E.V. 76			
	Actively immunized		Passively immunized	Not immunized	Actively immunized		Passively immunized	Not immunized
	Fraction I	Toxin			Fraction I	Toxin		
Cortisone*	20/20	20/20	20/20	5/20	20/20	20/20	20/20	20/20
No cortisone	20/20	20/20	20/20	20/20	20/20	20/20		10/10

* Dose of cortisone—2.5 mg/mouse, 4 hours prior to inoculation.
† Survivors/total inoculated.

congested and studded with light yellow to gray areas. Sections of the cortex and medullary portions revealed small hemorrhages. Albuminous debris in the glomerular capsule and the tubules was prominent. Erythrocytes had accumulated around the blood vessels of the interstitial framework of the kidneys. The adrenals were small and normal in color and architecture; in some mice the medulla was soft and hyperemic.

In normal mice untreated with cortisone, E.V. 76 infection resulted only in occasional small areas of necrosis in the spleen and liver, which contained polymorphonuclear leucocytes and monocytes.

The gross and microscopic lesions of cortisone-treated mice were characteristic of terminal *Past. pestis* infection produced by virulent strains. The large number of *Past. pestis* found in the blood and organs clearly indicates that the infections were widespread and massive. The disappearance of large numbers of organisms from the organs from the 3rd to 6th days probably represents lysis and release of massive amounts of toxin. The necrosis in liver and spleen and the cellular changes in the kidney indicate the extensive effects of the toxin in these infections with attenuated strains.

DISCUSSION

When the mouse host was under the influence of cortisone, some attenuated, ordinarily innocuous strains of *Past. pestis* produced a bacteremia and fatal infection very similar to that produced by a fully virulent strain. Other bacteria and viruses not ordinarily pathogenic or of low pathogenicity for a particular host may, when that host is under the influence of cortisone, produce an extensive, invasive infection ending in death.[10–14] That the increased mortality in the experiments reported here was due to *Past. pestis*, not to other latent organisms, is clearly indicated in the protection afforded by specific immunization and in the large numbers of *Past. pestis* in the blood and organs of inoculated mice treated with cortisone.

Particularly striking in the present study were the bacteremia produced and the large numbers of organisms in the tissues of cortisone-treated mice. Bacteremia also tends to develop during pneumococcal infection[15] or streptococcal infection[12,16] and cortisone administration. In previous experiments[6] with E.V. 76, although it regularly produced small centers of bacterial multiplication and necrosis in the spleen and liver of mice, even with colonies of organisms in such vascular organs, bacteremia or blood stream invasion was not demonstrable, and, after an initial phase of multiplication lasting approximately 4 days, these areas had disappeared. The effects of cortisone on infections do not appear attributable to direct action on the bacterial cell itself resulting in increased multiplication.[15–17] And, while evidence is accumulating that antibody production is inhibited by cortisone,[18,19]

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demonstrations of this have required that quite large quantities of the hormone be given over a period of at least several days. The effect of a single relatively large dose of cortisone on the antibody response to plague antigens in mice remains to be determined, but in view of the short duration of the infection in the present experiments and the fact that only a single dose of cortisone was given, it seems unlikely that antibody suppression was the prime factor in the extensive, invasive infection.

Local inflammatory reactions and infection-localizing processes seem clearly to be markedly inhibited by ACTH and cortisone.[17,20] Prominent among the alterations has been the failure in mobilization of leucocytes and other phagocytes at the site of infection.[12,15–17] In rodent hosts this inhibition of mobilization appears to be the predominant effect on phagocytes, rather than a decrease in number of circulating polymorphonuclear leucocytes or suppression of the phagocytic activity of individual phagocytes. The work of Martin and Kirby[21] and Germuth and associates[15] further suggests that it is failure in localization of infection, rather than alteration in the host's capacity for removal of bacteria from the blood, that results in the frequently observed bacteremia during infections accompanied by administration of cortisone. The data presented here seem to confirm previous evidence[9] that the natural defense mechanisms against *Past. pestis* infection are primarily cellular and depend on localization of the infection by derivatives of the reticulo-endothelial system in conjunction with the local mobilization of polymorphonuclear leucocytes.

There is not yet any clear explanation of the difference between "virulence" and "avirulence" in strains of *Past. pestis*. More than one factor may be involved, and different factors may be responsible for loss of virulence in different strains. There is, furthermore, evidence[2,3,6] that strains may lose their ability to produce rapidly fatal general infections, but through their toxin still produce local lesions. This characteristic is markedly in evidence for the E.V. 76 strain. It is of particular interest in the experiments described herein that although strains E.V. 76 and F7793-10 are capable of producing only a very limited infection in a normal mouse, the intervention of only a single injection of cortisone allows these strains to produce an infection with all of the characteristics of one by a fully virulent strain. On the other hand, even in a host tissue environment altered by cortisone and suitable for rapid multiplication of E.V. 76 and F7793-10, strains A1122 and Tjiwidej did not produce a comparable infection.

SUMMARY

After a single injection of cortisone the mortality rate of mice challenged with attenuated *Pasteurella pestis* strains E.V. 76 and F7793-10 was much higher than that among mice untreated with cortisone, and the degree of susceptibility was proportional to the dose of cortisone. *Past. pestis* bacteremia was definite in most cortisone-treated mice, and infection was widespread. Mice were protected from these effects by specific immunization. In the doses tested, cortisone did not significantly influence the susceptibility of mice to *Past. pestis* toxin. Cortisone had no effect on the mortality rate of mice infected with strains A1122 and Tjiwidej.

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PATHOGENESIS OF EXPERIMENTAL HISTOPLASMOSIS IN MICE

I. MEASUREMENT OF INFECTING DOSAGES OF THE YEAST PHASE OF *HISTOPLASMA CAPSULATUM*

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To study the pathogenesis of experimental histoplasmosis in laboratory animals it is desirable to accurately measure infecting dosages. Suspensions of yeast phase growth of most strains of *Histoplasma capsulatum* contain many multicellular aggregates of cells. Methods were developed for preparing suspensions containing only small aggregates of yeast cells and for determining the viability of small aggregates. Investigations using these methods include: a comparison of viability of yeast cell aggregates obtained from cultures of various ages, measurements of the survival of yeast cell aggregates in suspension, and a comparison of the in vitro viability of yeast cell aggregates with their infectivity for mice. The experimental procedures developed have been found to be applicable to four representative strains or isolates of histoplasma described in table 1.

MATERIALS AND METHODS

Yeast phase cultures of H. capsulatum.—Yeast phase cultures of each of the 4 strains were grown on slants of glucose cystine blood agar.[1] Each slant was inoculated with a loop of inoculum containing approximately 5 to 10×10^7 cells obtained from similarly inoculated 2-day-old cultures. The inoculated slants were cotton-stoppered and were incubated at 37 C in a sealed dessicator jar containing a water reservoir.

Preparation of yeast cells suspensions.—The total growth from 4 slants was washed off with 2 washings of 1.0 ml of 0.86% saline per slant. The cells were pooled and washed 2 times in approximately 50 ml of saline. After the last washing the cells were suspended in saline to a final volume of 32 ml. An 8.0 ml aliquot of the cell suspension was removed for growth determinations. The remaining suspensions was differentially centrifuged at 350 r.p.m. for 3 minutes in an angle head Servall type SP centrifuge. Fifteen ml of the supernate were carefully removed by aspiration. Dilutions of this supernatant suspension were used for making colony counts and for infecting mice.

Cell and aggregate counts.—Dosages were based on chamber counts done in Levi haemocytometers. Depending on the turbidity of the supernatant suspension, 2 dilutions of either 1:5, 1:10, or 1:20 were made using different 1.0 ml and 5.0 or 10.0 ml tip delivery serologic pipettes. Counts were done on each dilution in 2 to 4 chambers. In each chamber the cells per 0.1 cmm and the aggregates of cells in the same 0.1 cmm were counted; in each experiment more than 2000 cells and more than 1000 aggregates were counted. Aggregates represented budding cells which were not dissociated during the washing procedures. For the cell count, attached buds were counted as separate cells; for the aggregate count, single cells as well as aggregates were counted as aggregates. The number of cells per ml of the supernatant suspension was considered as the average of all chamber cell counts times the dilution counted times 10,000. The number of aggregates per ml was similarly calculated from the average of all chamber aggregate counts.

The effectiveness of eliminating the large aggregates by differential centrifugation was indicated by the average number of cells per aggregate, determined by dividing the total cell count by the total aggregate count.

The volume of inoculum for injecting mice and for inoculating flasks for colony counts was 0.5 ml.

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1. Rowley, D. A., Haberman, R. T. and Emmons, C. W. 1954, Histoplasmosis: pathologic studies of fifty cats and fifty dogs from Loudoun County, Virginia. J. Infect. Dis. 95: 98–108.

TABLE 1.—*History and cultural characteristics of 4 strains of* H. capsulatum.

Strain	History				Cultural characteristics			
	Date isolated	Original host	Conversion to yeast phase	Interval, conversion to experimental infections	Yeast phase growth	Mycelial phase growth*		In vitro conversion of mycelial phase to yeast phase†
						Micro-conidia	Macro-conidia	
6617	6-'49	Skunk	Mouse passage	30 months	Granular	Many	Few	None in 10 passages
6622	10-'52	Cat	Mouse passage	3 weeks	Mucoid	Few	Many	Conversion, 1 passage
6624	12-'52	Man	Mouse passage	3 weeks	Mucoid	Few	Many	None in 10 passages
6627	5-'53	Man	Transfer on glucose cystine blood agar	4 weeks	Granular	Many	Many	Conversion, 3 passages

* Description of cultures obtained from experimentally infected mice in these experiments.
† Transfer on glucose cystine blood agar every 3 days.

The dosages used to infect mice were an estimated 100, 4, 2, and 1 aggregates. In one experiment the death-producing effects of cells prepared from cultures of various ages were compared and the dosages tested were an estimated 2, 4, and 8×10^6 cells per mouse.

Dilutions were made in 0.86% saline unless otherwise indicated. In selected experiments 0.1% cysteine in water or saline was used for washing cells and/or for a diluent. The cysteine solution was adjusted to pH 6.5 with 2 *N* potassium hydroxide, sterilized by autoclaving, and stored in evacuated flasks.

Growth determinations.—The determinations were made on an 8 ml aliquot of the 32 ml of washed cell suspension. The 8 ml aliquot was centrifuged in a calibrated centrifuge tube at 4000 r.p.m. for 30 minutes in a horizontal head International Centrifuge, no. 1. The volume of packed cells was read to the nearest 0.01 ml. The packed cells were resuspended and dilutions were made for optical density and chamber count determinations. Optical density readings were made in a Beckman model B spectrophotometer at 660 mμ. Chamber cell counts were done as previously described.

Flask colony counts.—Flasks were used rather than petri dishes for making colony counts. Adequate humidity could be maintained and contamination almost eliminated by using flasks. The medium for making colony counts contained per liter: 10 g Casamino Acids (Difco), 20 g glucose, 15 g agar, 1 mg Ca pantothenate, and 0.5 mg biotin. The pH of the Casamino Acids and vitamin solution was adjusted to 6.5 with 2 *N* potassium hydroxide. Agar was added and the mixture was autoclaved at 15 lb for 15 minutes. Sterile dry glucose was added after the medium cooled to 60 C. Fresh medium was prepared 24 hours prior to each experiment. Twenty ml of medium were dispensed per 250 ml cotton-stoppered Erlenmeyer flask. The inoculum was distributed over the solid surface of the medium and the flasks were incubated at 30 C. The mycelial colonies in each flask were counted on the 7th, 9th, and 12th day after inoculation. The colony count recorded for each flask was the highest count obtained at any one of these time intervals.

Before beginning mouse injections 10 or more flasks were each inoculated with 0.5 ml of an aggregate suspension. After the completion of the mouse injections a set of flasks was similarly inoculated with the same suspension. Since the number of mice injected and the time required to inject the mice varied in each experiment, the second set of flasks was inoculated 2 hours after the first set of flasks.

Mice and cultures of mouse tissues.—General purpose Swiss White male mice, 5 to 6 weeks old and weighing 18 to 20 g, were used. The mice were distributed 4 mice to a jar and were fed water and Purina Chow ad libitum. Prior to intravenous injections the jars of mice were placed in a 37 C room for approximately 30 minutes to produce peripheral vasodilatation. Injections were made in a lateral tail vein, using a 0.5 ml tuberculin syringe with a ½", 27 gauge long bevel needle. Eighty to 100 mice could be injected in less than 2 hours.

All cultures of mouse tissues were made on slants of modified Sabouraud's glucose agar.*

Mice were sacrificed by chloroforming. Two sets of sterile instruments were used to expose the peritoneal and thoracic cavities. Six cultures were obtained from each mouse: 2 cultures each of the lungs, liver, and spleen. A separate set of sterile

* This medium contains per liter 10 g of Neopeptone (Difco), 20 g of glucose, and 20 g of agar. The pH of the medium is adjusted to 5.9. The combined components of the medium are autoclaved at 15 lb for 15 minutes.

instruments was used to obtain cultures of each tissue. A tissue sample approximately 4×4×4 mm was placed on the surface of the slant and macerated with the scissors. The tissues were streaked over the surface of the medium with a sterile inoculating needle, and the fragments were left scattered over the surface of the slant. The cultures were incubated at 30 C and were examined at frequent intervals; all cultures were held for 6 weeks prior to discarding. The mycelial cultures of histoplasma were identified by the characteristic gross and microscopic morphology.

EXPERIMENTS

1. Growth of the yeast phase of 4 strains of histoplasma on glucose cystine blood agar

Preliminary experiments demonstrated that for the 4 strains of histoplasma glucose cystine blood agar supported similar germination of isolated yeast cells to yeast cell colonies. In the following experiment the growth of these strains was compared on the glucose cystine blood agar using a large inoculum.

For each strain of histoplasma 12 slants were each inoculated with a loop of inoculum containing approximately 10^8 cells. For a given determination at 2, 4, and 8 days the total growth from 4 slants was pooled and cell volumes, cell counts, and optical density determinations were obtained on aliquots of the pooled samples. The cell count and optical density determinations for "day-O" were made on a loop of inoculum suspended in 10 ml of saline; for each strain this determination was done in quadruplicate. The results of the growth studies are presented in table 2. The figures for cell volumes, cell counts, and optical densities for 2-, 4-, and 8-day-old cultures are equivalent to the average growth per slant.

The results of these studies demonstrated that the 4 strains of histoplasma grew similarly on glucose cystine blood. agar. With a large inoculum the rapid growth phase occurred during the first 48 hours, though for each strain growth continued through the 4th and 8th days.

2. Viability and death-producing effects of aggregates prepared from cultures of various ages

Suspensions were prepared from aliquots of the 2-, 4-, and 8-day-old cultures of strain 6617 used for the growth determinations presented in table 2. The average number of cells per aggregate was 2.1 cells for each of these 3 supernatant suspensions. Dilutions were made of each suspension to contain per 0.5 ml an estimated 8×10^6 cells, 4×10^6 cells, 2×10^6 cells and 100 aggregates. Flasks were inoculated with an estimated 100 aggregates per flask in order to compare the viability of aggregates obtained

TABLE 2.—*Growth of 4 strains of* H. capsulatum *on glucose cystine blood agar.*

	Age of cultures in days	Strains of histoplasma			
		6617	6622	6624	6627
Packed cells, volume in ml	0	N.D.	N.D.	N.D.	N.D.
	2	0.09	0.10	0.10	0.10
	4	0.19	0.28	0.28	0.20
	8	0.25	0.35	0.35	0.30
Cell counts $\times10^2$	0	8–11	8–10	7–9	7–9
	2	292	289	268	162
	4	1080	1386	1180	1240
	8	1220	1680	1470	1380
Optical densities	0	0.9–1.2	0.7–0.9	0.5–0.7	0.7–0.9
	2	25	18	18	15
	4	70	78	82	77
(O.D. ×1/dilution)	8	87	97	104	111

N.D. =not done.
O.D. =optical density.

from cultures of various ages. Groups of mice were infected with the three large dosages in order to compare the death-producing effects of cells obtained from cultures of various ages.

The results of the flask counts are presented in table 3. The data show that there were about 50% as many viable aggregates in the suspension prepared from the 8-day-old cultures as there were in the suspension prepared from the 2-day-old cultures. There was also decreased vitality of aggregates prepared from the older cultures, as indicated by comparing the survival of aggregates in saline suspension for 2 hours. About 87% of the aggregates prepared from 2-day-old cultures and only 22% of the aggregates prepared from 4- and 8-day-old cultures survived in saline suspension for 2 hours.

The relation between age of culture, dosage, and deaths of infected mice is presented in table 4. The increase in the number of surviving mice infected with suspensions prepared from the older cultures reflects the decreased viability and vitality of aggregates in these suspensions as measured by flask colony counts.

TABLE 3.—*Colony counts on flasks inoculated with an estimated 100 aggregates prepared from cultures of various ages.**

Flask†	2-day-old cultures		4-day-old cultures		8-day-old cultures	
	0	2 hr.	0	2 hr.	0	2 hr
1	90	84	67	17	52	13
2	86	80	66	16	51	12
3	85	76	66	16	48	12
4	84	76	66	15	45	10
5	83	72	63	14	43	10
6	80	69	62	12	41	10
7	79	67	60	10	39	7
8	73	63	59	10	38	7
9	71	61	50	10	36	6
10	70	60	40	9	34	5
Average number of colonies per flask	80	71	60	13	43	9
Average number of cells per aggregate	2.1		2.1		2.1	

0—flasks inoculated prior to mouse injections.
2 hr—inoculum held in saline at room temperature 2 hours.
* Strain 6617 of *H. capsulatum*.
† Flasks arbitrarily arranged and numbered in order of decreasing numbers of colonies.

TABLE 4.—*Relation between age of culture, dosage, and deaths of infected mice.**

Age of cultures	Dosage of yeast cells: Number of cells ×10⁶	Dosage of yeast cells: Number of aggregates ×10⁶	Mice: Average weight in g	Mice: Time 50% of mice were dead	Mice: Number of mice dead at 30 days
2 days	8	3.8	18.0	13 days	20/20
	4	1.9	18.2	26 days	12/24
	2	0.95	18.3	30 days	12/23
4 days	8	3.8	18.6	15 days	17/20
	4	1.9	18.3	>30 days	8/20
	2	0.95	18.6	>30 days	5/20
8 days	8	3.8	18.6	18 days	16/22
	4	1.9	18.5	>30 days	7/22
	2	0.95	18.2	>30 days	3/21

* Strain 6617 of *H. capsulatum*.

The rapid loss of viability of aggregates in saline suspension has been found for suspensions prepared from either heavily inoculated or old cultures of strains 6622, 6624, 6627, as well as for strain 6617. The possibility that the loss of viability of aggregates in suspension was only apparent and due to saline agglutination of aggregates was investigated. By direct microscopic examination no agglutination of aggregates was demonstrated in saline or on the surface of the medium used for making colony counts.

3. Viability and infectivity of an esimated 100 aggregates prepared from 2-day-old cultures of 4 strains of histoplasma

Suspensions containing an estimated 100 aggregates per 0.5 ml were prepared from 2-day-old cultures of each of the 4 strains of histoplasma. The cultures of each strain were in the same phase of growth as the 2-day-old cultures used for the growth determinations in table 2. For each strain 20 to 22 mice were injected intravenously with 0.5 ml of a suspension, and flasks for colony counts were inoculated with 0.5 ml of the same suspension. The mice were sacrificed 4

TABLE 5.—*Colony counts on flasks inoculated with an estimated 100 aggregates prepared from 2-day-old cultures of 4 strains of* H. capsulatum.

Flask*	Strains of histoplasma			
	6617	6622	6624	6627
1	83	84	78	91
2	81	73	72	90
3	80	65	70	81
4	79	65	69	78
5	77	64	66	74
6	76	64	60	68
7	75	63	56	66
8	75	60	56	64
9	73	59	53	61
10	66	58	53	60
11			53	
12			50	
Average number of colonies per flask	77	66	60	73
Average number of cells per aggregate	2.0	1.5	1.4	1.7

† Flasks inoculated prior to mouse injections.
* Flasks arbitrarily arranged and numbered in order of decreasing number of colonies.

to 6 weeks after the intravenous injections.

The results of the colony counts are presented in table 5. The counts in each experiment are for the 10 to 12 flasks inoculated prior to the beginning of the mouse injections. For each strain of histoplasma there were 10 to 20% fewer colonies on the flasks inoculated 2 hours after the initial set of flasks. The average number of cells per aggregate in the 4 suspensions ranged from 2.0 cells for strain 6617 to 1.4 cells for strain 6624. For each strain more than 60% of the aggregates consisted of 2 cells or single cells. The average number of colonies per flask ranged from 60 for strain 6624 to 77 for strain 6617.

Histoplasma was isolated from 22 of 22 mice infected with strain 6617, from 21 of 22 mice infected with strain 6622, from 20 of 20 mice infected with strain 6624, and from 20 of 20 mice infected with strain 6627. All cultures of tisues of one mouse infected with strain 6622 were contaminated, and histoplasma was not isolated from that mouse.

For each of the 4 strains of histoplasma (1) suspensions of small aggregates were obtained, (2) comparable flask colony counts were obtained, and (3) infections in mice were uniformly induced by the intravenous injection of an estimated 100 aggregates.

4. Survival of aggregates of yeast cells of histoplasma in suspension

The time required to inject 80 to 100 mice intraveously was about 1½ to 2 hours. The elapsed time from the beginning of harvesting of cultures to the completion of mouse injections was about 4 hours, and the time required for these procedures could not be reduced appreciably. The results of the flask counts made before beginning mouse injections and after a 2-hour interval suggested that the number of viable aggregates in a suspension was not constant during the time required to inject a large number of mice. Because of these findings experiments were designed to test factors which might affect survival of aggregates in suspension.

Preliminary experiments demonstrated that there was a similar loss of viable aggregates over a 2-hour interval in suspensions made in distilled water, 0.85% sodium chloride, or 5% dextrose. However, there was no apparent loss of viable aggregates during the same interval in aliquots of these suspensions stored at 4 C or in suspensions made in 0.1% cysteine in water and held at room temperature.

The yeast cells from 2-day-old cultures of strain 6617 were harvested with saline at 4 C. The yeast cells were washed in cold saline and the supernatant suspension was held at 4 C while chamber counts were made. The aggregate suspension was diluted in cold saline to contain 10^6 aggregates per 0.5 ml. Dilutions of this suspension were made in (1) 0.1% cysteine in saline at 4 C, (2) saline at 4 C, (3) 0.1% cysteine in saline at 24 C, and (4) saline at 24 C, so that the final dilutions of each suspension contained an estimated 100 aggregates per 0.5 ml. For each suspension 8 flasks

were inoculated with 0.5 ml of suspension per flask. At 2-, 4-, and 24-hour intervals additional sets of 8 flasks were similarly inoculated with each of the 4 suspensions. The 2 suspensions made in cold solutions were held at 4 C for the 24-hour period; the other 2 suspensions were held at 24 C for the 24-hour period.

The results of the colony counts on the flasks inoculated with the 4 suspensions are presented in fig. 1. Each point on the graph is the average colony count for 8 flasks. The results show that during a 4-hour period the number of viable aggregates in suspension remains constant in either saline at 4 C or in 0.1% cysteine at 4 C. There was appreciable loss of viable aggregates during the same period in the 2 suspensions held at 24 C. At the end of 24 hours the number of viable aggregates in the suspension in 0.1% cysteine in saline at 4 C remained high, while there was a marked loss of viable aggregates in the other 3 suspensions.

Strain 6624 was tested in the same way, except that the original suspension was made in saline plus 0.1% cysteine. Results were similar for this strain. Colony counts on suspensions prepared in cold solutions were similar to the initial counts obtained on suspensions prepared at room temperature. Apparently the loss of viability of aggregates in suspension at room temperature occurs more than 2 hours after the yeast cells are harvested from cultures.

5. The colony count as a measure of the number of infective aggregates in a suspension

In more than 20 consecutive experiments using suspensions of 2-day-old cultures of 4 strains of histoplasma, the average of the flask colony counts was 49 to 80% of the estimated 100. In more than 10 subsequent experiments similar results were obtained for suspensions prepared in cold saline or in cold saline with cysteine. It appeared probable that there were aggregates in these suspensions which did not germinate and grow to macroscopic mycelial colonies on the medium used, yet might be infective for mice.

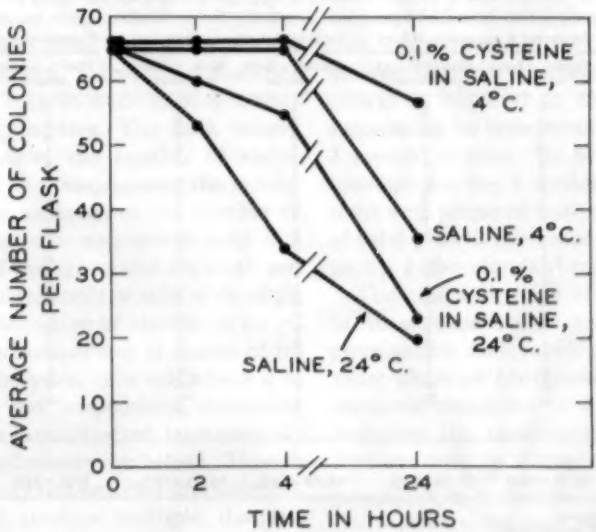

FIG. 1.—Survival of aggregates of *H. capsulatum*, strain 6617, in suspensions at 4 and 24 C.

An experiment was designed to test whether the colony count is an adequate measure of the number of infective aggregates in a suspension. For this experiment the cells from 2-day-old cultures of strain 6624 were washed in cold saline, dilutions were made in cold saline, and the dilutions were held at 4 C during the time required to inoculate flasks and inject mice. The average number of cells per aggregate was 1.6 cells, and a differential count on the number of cells per aggregate showed that 84% of the aggregates consisted of 2 cells or single cells.

A suspension containing an estimated 100 aggregates per 0.5 ml was prepared. Twenty flasks were each inoculated with 0.5 ml of this suspension. Three dilutions of the suspension were made to contain per 0.5 ml an estimated 4, 2, and 1 aggregates. For each of these 3 dilutions 22 replicate flask cultures were inoculated with 0.5 ml of suspension per flask, and 22 to 24 mice were injected intravenously with 0.5 ml of suspension per mouse. The mice were sacrificed 6 weeks after the intravenous injections.

The results of the flask colony counts and the cultures of mouse tissues are presented in table 6. The total colony count for the 61 flasks inoculated with an estimated total of 143 aggregates was 77. The average colony count for the 20 flasks each inoculted with an estimated 100 aggregates was 50. It is apparent that the germination of aggregates and the technique for counting colonies is not appreciably affected by the number of aggregates inoculated per flask.

Histoplasma was isolated from 44 of the 69 mice. Histoplasma was isolated from all cultures of the livers and spleens of these 44 mice. Only 4 cultures of lungs from 4 mice were contaminated. It is unlikely that the results of the experiment were altered by contaminated cultures of mouse tissues or inadequate sampling of possibly infected tissues.

For each dosage the percentage of mice infected was approximately the

TABLE 6.—*Comparison of in vitro viability of aggregates with their infectivity for mice.**

Flask or mouse†	Estimated 4 aggregates/0.5 ml		Estimated 2 aggregates/0.5 ml		Estimated 1 aggregate/0.5 ml	
	Flasks, colony counts	Mice, cultures of tissues	Flasks, colony counts	Mice, cultures of tissues	Flasks, colony counts	Mice, cultures of tissues
1	6	+	2	+	2	+
2	4	+	2	+	2	+
3	4	+	2	+	1	+
4	3	+	2	+	1	+
5	3	+	2	+	1	+
6	3	+	1	+	1	+
7	3	+	1	+	1	+
8	3	+	1	+	0	+
9	3	+	1	+	0	0
10	3	+	1	+	0	0
11	2	+	1	+	0	0
12	2	+	1	+	0	0
13	2	+	1	+	0	0
14	2	+	1	+	0	0
15	2	+	0	+	0	0
16	1	+	0	+	0	0
17	1	+	0	0	0	0
18	1	+	0	0	0	0
19	1	+	0	0	0	0
20	0	+	x	0	0	0
21	0	0	x	0	0	0
22	x	0	x	0	x	0
23				0		0
24						0
Percent of flasks or mice with cultures of *H. capsulatum*	19/21 = 90%	20/22 = 90%	14/19 = 74%	16/23 = 70%	7/21 = 33%	8/24 = 33%

x = flasks contaminated.
* Strain 6624 of *H. capsulatum*.
† Flasks arbitrarily arranged and numbered in order of decreasing numbers of colonies. Mice arbitrarily arranged and numbered in order of presence or absence of cultures of histoplasma.

same as the percentage of flasks with colonies. It is not possible to determine how many aggregates were injected into a particular mouse; however, it is probable that the mice received approximately as many viable aggregates as were inoculated on corresponding flasks. Among the 61 flasks inoculated with an estimated 1 to 4 aggregates, 18 flasks had single colonies. The data suggest that a single viable aggregate is sufficient to infect a mouse and that the flask count technique is an accurate measure of the number of infective aggregates in a suspension prepared from 2-day-old cultures of strain 6624.

DISCUSSION

One of the objectives of these studies is to define a method of comparing the pathogenicity of various isolates or strains of histoplasma. The 4 strains used were purposely selected because of their varied histories and cultural characteristics. The pathogenicity of these strains in mice and in other laboratory animals will be presented in subsequent papers.

Several considerations stimulated investigations of methods for obtaining suspensions of small aggregates of cells of histoplasma. Accurate chamber counts can only be made on suspensions of small aggregates. The flask colony counts measure the number of viable aggregates in a suspension; the colony counts give an index of the number of viable cells in a suspension only if a majority of the aggregates are small and consist of single cells or cells with single buds. An objective of studies to be reported is a comparison of routes of infection. The yeast cells are 4 to 5 μ in diameter, and suspensions containing large aggregates injected intravenously produce pulmonary embolism. Though the mice may not be killed immediately, the emboli produce multiple, discrete, hemorrhagic pulmonary infarcts. These infarcts are not produced by very large dosages of small aggregates injected intravenously. A number of different methods of homogenization and filtration were tried for obtaining suspensions of small aggregates. Differential centrifugation proved to be the most simple, rapid, and reproducible method for obtaining satisfactory suspensions.

Suspensions prepared from the granular strains such as 6617 and 6627 contain many large aggregates of 10 or more cells. For these strains this type of yeast phase growth was present in a variety of solid and liquid mediums, and it was not prevented by varying the temperature of incubation or adding a "wetting agent" to the medium. The technique of differential centrifugation for obtaining suspensions of small aggregates was devised for obtaining suspensions suitable for producing overwhelming fatal infections as well as minimal infections in mice. For each of these strains more than 10^6 small aggregates injected intravenously are necessary to produce overwhelming fatal infections in mice. For the granular strains such as 6617 and 6627 about 80% of the cells are sedimented in the process of differential centrifugation. More total growth is required to obtain suitable suspensions for these strains than for the 2 mucoid strains. To keep conditions constant for the 4 strains, cell suspensions were prepared from equal amounts of total growth harvested from cultures grown under standard conditions.

The glucose cystine blood agar used in these experiments supports better germination and rapid growth of the yeast phase of histoplasma than other mediums compared in this laboratory, including the modification of Francis' medium used by Campbell.[2]

2. Campbell, C. C. 1947, Reverting *Histoplasma capsulatum* to the yeast phase. J. Bact. **54**: 263–264.

During the rapid growth phase of histoplasma on glucose cystine blood agar the generation time is about 8 hours. It is necessary that the cultures be incubated for more than 24 hours in order to prepare suspensions which do not contain a large percentage of cells from the original inoculum. The total growth harvested from 4 slants, each inoculated with 10^8 yeast cells of the granular strain 6617 and incubated for 48 hours, was just sufficient for adequate growth determinations and the preparation of an aggregate suspension for mouse infections. This same sized inoculum and time of incubation was adopted for the other strains in order to keep conditions constant for all strains.

The Casamino Acids medium, though not a synthetic medium, was developed as an outgrowth of nutritional studies on a synthetic, chemically defined medium which supports "optimal" germination and growth of isolated yeast cells to mycelial colonies.[3] The Casamino Acids medium is inexpensive and easily prepared. The addition of agar to the medium does not inhibit germination or growth of the organism. The addition of as little as 0.1% KH_2PO_4 to the agar-containing medium markedly inhibits germination and growth. This may explain why the addition of agar to another Casamino Acids containing medium described for growing the yeast phase of histoplasma[4] is not satisfactory for colony counts.

In repeated experiments using different strains of histoplasma, colony counts made on flasks of glucose cystine blood agar incubated at 30 C were more than 50% greater than counts made on companion flasks incubated at 37 C. For these studies the germination of yeast cells to yeast cell colonies at 37 C. was not an adequate measure of the number of viable aggregates in a suspension. The Casamino Acids medium does not support good yeast phase growth; however, the medium supports as good germination and growth of isolated yeast cells to mycelial colonies at 30 C as the glucose cystine blood agar medium. The Casamino Acids medium is clear and colorless, and the colonies can be readily counted through the bottom of the flasks; colonies are more difficult to count on the opaque blood containing medium.

There is no evidence that flask cultures are seeded by spores from growing mycelial colonies. In the last experiment 40 flasks, each with 1 to 6 colonies, were held for 4 weeks; the colonies were counted on the 7th, 9th, and 12th days, and no additional colonies appeared during the following 2 weeks.

Multiple cultures of histoplasma have been obtained from mice injected intravenously with fewer than 100 viable aggregates and sacrificed 1 to 2 hours later. Modified Sabouraud's glucose agar does not support good germination and growth of isolated yeast cells. The addition of tissue fragments to the slants apparently make it an adequate medium for demonstrating the presence of a very few yeast cells in mouse tissues.

It is unlikely that the results of these studies have been affected by cross infections of mice housed in the same jar. In the last experiment 69 mice were distributed in 18 jars; mice with sterile cultures were obtained from 13 of the jars. In 2 preliminary experiments noninfected mice were placed in jars with heavily infected mice for 6 weeks. No cross infections were found.

Certainly mice are very susceptible to infections induced by the intravenous injection of a few yeast cells of histoplasma. These findings are consistent

3. Rowley, D. A. Unpublished data.
4. Salvin, S. B. 1950, Growth of the yeast like phase of *Histoplasma capsulatum* in a fluid medium. J. Bact. **59**: 312–313.

with those of Ajello et al,[5] who worked with the mycelial phase. Using single spore isolation technique, they were able to induce infections in mice by the intraperitioneal implantation of single macroconidia.

The last experiment was designed to test whether the colony count is a measure of the number of infective aggregates in a suspension. For each dosage level a chi-square test of the agreement between the number of flasks with colonies and the number of mice infected shows that by chance alone a disagreement as great or greater could be expected about 90% of the time. Analysis of the data also supports the suggestion that a single viable aggregate is sufficient to infect a mouse. If 1 viable aggregate is sufficient to infect a mouse, denote by Pi the proportion of mice infected at the "i"th dilution ($i=1,2,3$) of inoculum and denote by λi the average concentration of viable aggregates per 0.5 ml at the "i"th dilution. Approximately, $Pi=1-e^{-\lambda i}$ where e is the base of the natural system of logarithums ($e=2.718$), providing the suspension of inoculum is well mixed and the volume of an individual aggregate is small compared to the volume of inoculum administered. With these assumptions the theoretical number of mice infected at each dosage level can be computed; the agreement between the theoretical number of mouse infections and the observed number of mouse infections can be tested. The average number of viable aggregates for each dosage was determined by the method outlined by Fisher and Yates.[6] Omitting the computations, the following comparison is obtained:

Estimated dosage of aggregates	Average number of viable aggregates for each dosage	Observed number of mice infected	Theoretical number of mice infected
4	1.98	20/22	18.95/22
2	0.99	16/23	14.44/23
1	0.49	8/24	9.36/24

A chi-square test of agreement between the observed and the theoretical number of mice infected shows that by chance alone discrepancies as large or larger than those observed could occur more than half the time. This lends considerable support to the suggestion that one viable aggregate is sufficient to infect a mouse.

The arbitrary "infecting dose" for most experimental purposes has been an estimated 100 aggregates. It is of interest that young mice weighing 8 to 10 g infected with an estimated 100 viable aggregates mature and reproduce normally. No gross lesions are produced; however, the organism can be regularly isolated from these mice as long as 6 months after the induction of infection.

SUMMARY

Four strains of histoplasma were grown in the yeast phase on a complex whole blood containing medium under standardized conditions. A technique for obtaining suspensions of small aggregates of yeast cells and a method for determining the in vitro viability of aggregates are described. Conditions for obtaining suspensions containing a high percentage of viable aggregates and for maintaining a constant number of viable aggregates in a suspension are described. Data are presented which strongly suggest that under these conditions an in vitro test for viability is an accurate measure of the number of infective aggregates in a suspension.

5. Ajello, L. and Runyon, L. 1953, Infections of mice with single spores of *Histoplasma capsulatum*. J. Bact. 66: 34–40.
6. Fisher, R. A. and Yates, F. 1953, Statistical Tables for Biological, Agricultural and Medical Research, New York, Hafner Co.

EXPERIMENTAL TRANSMISSION OF VESICULAR STOMATITIS VIRUS BY DIPTERA

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Theiler[1] as early as 1897 demonstrated that the virus of vesicular stomatitis was transmitted from one horse to another through minute abrasions of the mucous membrane. He considered the rough feed of some range animals to be a factor in producing these sites of entry. Scratches on the teats of cattle were observed by Cotton[2] to be important in cases of the spread of the virus by the hands of milkers. On the basis of the difficulty of transmission, Cotton concluded that foot and mouth disease which spreads rapidly by contact was "on the opposite end of the scale" from vesicular stomatitis.

Although the virus was often transmitted with difficulty in the laboratory, French[3] observed that it could traverse great distances in wooded country and overcome a lack of communication between herds. The occurrence of the disease on isolated farms was well documented in the New Jersey outbreak of 1927 by Cotton[2] and in the 1942 epizootic in Colorado[4]. In regard to the latter, Heiny[4] wrote "The virus appears to be disseminated by means other than direct contact with affected animals, as it appeared in all these outbreaks on widely separated premises and that there had been no known contact of any nature by animals, trucks, or otherwise."

Heiny[4] went on to imply the possibility of vectors in his discussion of the bearing of topography and climate on the disease. Radeleff[5] in 1949 was apparently the first to suggest that the disease might be transmitted by an insect when he wrote that "the vector, if such existed, most suspected in these cases would be the stable fly, since these flies were present in hordes in and about the stables in question." In 1952, Hanson[6] reviewed the natural history of vesicular stomatitis and on the basis of the need for abrasions for entry of the virus and on the lack of direct contact between cases the agency of a vector was suggested. Among the vectors considered were the horseflies, deerflies, mosquitoes, and black flies.

This paper concerns attempts to transmit vesicular stomatitis by representative diptera.

MATERIALS AND METHODS

Virus.—The vesicular stomatitis virus, New Jersey serotype, Wisconsin strain (VSV-NJ-W), which was isolated in 1949[7] and cultivated since that time in embryonating chicken eggs, was used. Aliquots of allantoic fluid containing virus of the 10th to 15th embryo passage had an LD_{50} for chicken embryos of $10^{4.0}$ to $10^{6.5}$. The virus was stored at −20 C and when thawed for preparation of inoculums, it was kept in icewater.

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2. Cotton, W. E. 1927, Vet. Med. 22: 169–175.
3. French, A. W. 1918, J. Am. Vet. M. A. 52: 421–422.
4. Heiny, E. 1945, N. Am. Vet. 26: 726–730.
5. Rodeleff, R. D. 1949, Vet. Med. 44: 494–496.
6. Hanson, R. P. 1951, Bact. Rev. 16: 179–204.
7. Brandly, C. A., Hanson, R. P. and Chow, T. L. 1951, Proc. Book Am. Vet. M. A. 61–67.

Dilutions were usually made in nutrient broth or in liquid thioglycollate medium. Inoculation was into the allantoic chamber, and the allantoic fluid containing the virus was harvested when the embryo died usually 24 to 48 hours after inoculation. Sterility tests on each harvest of egg fluids were made by streaking the fluids on agar slants or by inoculation into fluid thioglycollate medium. Harvested material known to be contaminated with bacteria was mixed with antibiotics in proportions to allow 250 units of penicillin and 250 μg of streptomycin per 0.1 ml of inoculum.

Serum neutralization tests (SN) for the identification of virus obtained from vector infected eggs were performed by inoculating 4 or 5 eggs with a 10^{-1} dilution of suspected virus fluid in an antibiotic broth and another set of the same number of eggs with the same dilution of virus mixed with VSV bovine antiserum which had a neutralization titer of 10^{-2} to 10^{-3} LD_{50}. The bovine antiserum had been inactivated at 56 C for a half hour. The virus-serum mixture was incubated at room temperature for a half hour before injection into embryos.

Neutralization titers of antiserums were determined by mixing equal quantities of undiluted serum and decimal dilutions of virus. Death of the embryo was evidence of infection and survival for 4 days, of neutralization. The difference between the titer of the virus with and without the presence of antiserum determined the neutralizing titer of the serum.

Vectors.—The ability of representative diptera to transmit VSV experimentally was surveyed using individuals captured in north central Wisconsin. Most of the collections were made in the vicinity of Bruce, which is in the center of the area involved in the 1937 and 1949 vesicular stomatitis epizootics. Horseflies and deerflies were collected in barns from and around cows at pasture and from woods near farms. An aspirator picked off flies as they alighted on animals to bite. Many black flies and mosquitoes were obtained in the same way. A light trap described by Du Toit[8] assisted materially in making the mosquito collection.

Transportation of the flies to the laboratory was either in standard insect cages or in smaller ones improvised from cheesecloth and wire. Slices of orange, apple, or potato, and acetate sponges soaked in fruit juices furnished moisture and nourishment and apparently prolonged the life of the flies in captivity.

Laboratory-reared stable flies *Stomoxys calcitrans* Linn.[9] and mosquitoes, *Culex pipiens** Linn. and *Culex quinquefasciatus*† Say, were used for certain studies. Mosquito larvae were hatched in 3 inches of water held in enamel pans and fed ground mouse pellets. As the pupae formed they were transferred to glass emergence jars. Two sizes of insect cages were used for adults, a small one, 12 inches in diameter by 12 inches high, as a holding cage, and a large one, 18 inches in diameter by 18 inches high, as a breeding cage. Adults were fed fruit slices, soaked raisins, dextrose solution and cow's blood. Females that laid eggs were sometimes fed on chickens, guinea pigs or embryonating eggs. A large Petri dish with damp cotton covered with paper toweling served as a depository for egg rafts although a few were laid on the surface of the water. Egg rafts were transferred immediately to rearing pans or stored at 4 C for less than 5 days. Temperature in the insectory was kept around 26 to 27 C and the relative humidity as measured by the wet bulb was held about 45%.

Animal hosts.—Vesicular stomatitis virus indicates its presence in the natural host by the appearance of vesicular lesions in the oral mucosa, coronary band or teats.[6] Lesions are usually induced only when the virus is introduced at these sites. A satisfactory laboratory animal for transmission tests with insects presented a real problem. The guinea pig develops lesions on the footpads after intradermal inoculation of the virus. Intracerebral inoculation of mice, hamsters and ferrets produces a lethal infection. Clinical disease is not produced in laboratory animals by inoculation of the virus into the skin areas usually attacked by insects.

The report of Haas and Ewing[10] on the transmission of *Plasmodium gallinaceum* Brumpt to the 10- or 12-day-old chicken embryo suggested the use of the embryo as a host for testing the ability of the various insects to transmit the virus.[11] Embryonating eggs of 7 to 10 days are readily susceptible to vesicular stomatitis virus; the embryos dying within 24 to 48 hours. The specificity of the death may be determined by making sterility and serum neutralization tests. Stable flies, horseflies, deerflies, and mosquitoes bite the shell membrane in satisfactory numbers.

8. Du Toit, R. M. 1944, Onderstepoort J. Vet. Sc. **19**: 7.

9. Doty, A. E. 1937, J. Econ. Ent. **30**: 367–369.

* Collected in the vicinity of Madison.

† Eggs furnished by Bureau Ent. and Plant Quarantine USDA and Public Health Service.

10. Haas, V. H. and Ewing, F. M. 1945, Pub. Health Rep. **60**: 185.

11. Ferris, D. H. and Hanson, R. P. 1951, Cornell Vet. **42**: 389–394.

FIG. 1.—Horsefly in plastic egg tube.

FIG. 2.—Scotch tape air sac seal in use with black fly.

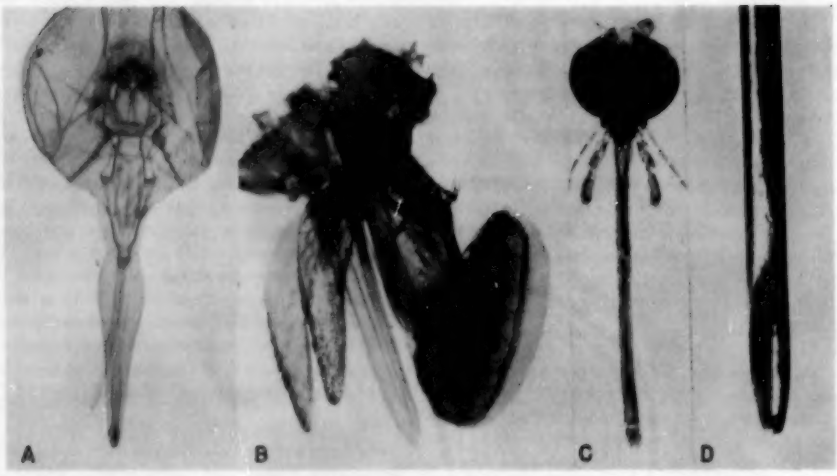

FIG. 3.—Composite photomicrograph, mouthparts of (A) stable fly, (B) horsefly, (C) mosquito in comparison with (D) 27 gauge hypodermic needle. All photomicrographs at ×18.

The embryonating egg was therefore the laboratory host of choice.

Twenty-four hours after the embryo was inoculated with the virus the insect was placed in the air sac chamber and left for intervals of an hour to a day. When the shell egg-membrane was found to be punctured, the insect was removed and held for 12 to 24 hours and then transferred to susceptible 8- or 9-day-old eggs.

The insect was left upon the susceptible embryo for 24 hours and then transferred to another embryo host at each succeeding 24-hour period. A small number of stable flies and mosquitoes rapidly engorged upon egg fluids. When this was noticed, feeding was interrupted. Most of the insects attacking the membrane, particularly the horse and deerflies interrupted their feeding and did not readily engorge. Transfers were usually continued until the death of the insect.

Temperature and humidity requirements were necessarily adjusted to the demands of the virus, insect, and embryo. A temperature of 35 C was satisfactory for the virus and embryo, but too high for proper longevity of the insect. By taking the eggs out of the incubator for several hours at a time, both embryos and insects were kept alive during the required period.

Two techniques were employed to restrain the insects over the air sac. Plastic tubes of various lengths were fabricated from centrifuge tubes by drilling a small slit in the top of the tube to allow satisfactory ventilation (fig. 1). A factor in securing puncture of membrane was the size of tube or enclosure. Insects kept down on the membrane made more punctures than those which were allowed to remain in a tube that permitted them to get some distance away. The variation which accomplished this consisted of covering the top of the air space with a piece of scotch tape upon which another had been placed, emulsion-to-emulsion. This method was fairly rapid and satisfactory for mosquitoes, stable flies and black flies but not for deerflies and horseflies, which died rapidly when confined in such a small space.

Other methods of infecting insects[12,13] were employed. Small tubes of blood were made with gut, frogskin, or placental membranes and insects allowed to feed on them. A Petri dish filled with blood-soaked cotton or gauze and sprinkled with sugar would induce tabanids, stable flies, and mosquitoes to feed at times. Horseflies could sometimes be fed with an eye-dropper or with a ground-off 27 gauge hypodermic needle, introduced into the mouthparts.

12. Reeves, W. C. and Hammon, W. M. 1945, J. Exper. Med. **83**: 185.
13. Reeves, W. C. and Hammon, W. M. 1952, J. Immunol. **69**: 511–514.

On occasion, when direct feeding of blood failed to give the desired results, insects were allowed to feed on chickens 3 to 5 weeks old which had been injected intravenously with 5 to 10×10^8 embryo LD_{50}'s of virus. The virus could be readily recovered from the blood 2 hours later and usually, but not always, 4 hours later.

In one experiment two cows from the University herds were used. Horseflies were placed on the cows in individual plastic tubes ¾ inch in diameter and 4 inches long. A piece of sponge was inserted in the bottom and gauze stretched over the open end of the tube. A small hole drilled in the bottom allowed the introduction of orange juice. Horseflies could be kept in the feeding tube and would remain alive for more than a week. Stable flies and mosquitoes were put in half pint mason jars, covered with copper screen to allow access to the animal. Both mosquitoes and stable flies bit through the screen on preliminary trials. A sponge in the bottom of the jar maintained the proper humidity and access to moisture without entangling the wings of the insects.

RESULTS

Survey of vector potential.—Vesicular stomatitis virus was transmitted from infected chicken embryos to susceptible embryos by representative species of several genera of diptera (table 1). One or more successful transmission attempts were obtained for 6 species of horseflies, 3 species of deerflies, and 3 species of mosquitoes. Under similar conditions, two species of horseflies, 3 of

TABLE 1.—*Species of captured diptera which transmitted vesicular stomatitis virus from infected to susceptible chicken embryos.*

Vector	Number of successful transmissions*
Tabanus lasiophthalmus Macquart	5
trispilus Weideman	1
lineola var *scutellaris* Walker	1
epistates Osten Sacken	6
affinis Kirby	1
trepidus McDonough	2
spp.	9
Chrysops montana Osten Sacken	2
striata Osten Sacken	6
villata Weidemann	1
spp.	1
Aedes stricticus (Meigen)	6
stimulans Walker	3
Culex tarsalis Coquillett	1
spp.	1

* Transmission from infected to susceptible embryos was accomplished by vectors which fed through the shell membrane, first of the infected and then, after 12 or more hours, of the susceptible embryo. Virus infection was determined by death of the embryo, freedom of the embryonic fluids from bacterial contamination and neutralization with antiserum of the infectivity of the embryonic fluids.

TABLE 2.—*Length of the period during which diptera infected susceptible chicken embryos after feeding on infected embryos.*

Vector	Hours elapsing between the exposure of the vector and exposure of the susceptible embryo to the vector								
	24			48			72		
	−	±	+	−	±	+	−	±	+
Stable fly	13	2	4						
Black fly	5	2	1	2			2		
Horn fly		3							
Horsefly	21	15	12	7	7	12	3	1	1
Deerfly	14	10	10	1				2	
Mosquito	19	9	9	2	4				2

− Number of embryos which failed to die or if they died were considered nonspecific.
± Number of embryos which died, usually without contamination but SN test of the fluid was negative or equivocal.
+ Number of embryos which died without contamination and SN tests of the fluids were positive.

deerflies and 3 of mosquitoes failed to transmit the virus. But an equal opportunity for transmission was not given each species as the number of individuals available for test differed. As a rule, fewer individuals were tested of the nontransmitting species. A transmission attempt was considered successful only if virus which could be neutralized by immune serum and which was free of bacterial contamination was isolated from the recipient egg.

Table 2, which presents the negative, equivocal and positive transmission attempts at 24, 48 and 72 hours after exposure of the vector, gives a better picture of transmitting ability of the groups of insects than a statement of success or failure. The equivocal results might have been positive if the identifying tests could have been run soon after the trial. As it was, contamination with bacteria or other factors prevented the inclusion of these data in the positive results. While several groups of diptera were capable of transmitting the virus for at least a day after feeding on infected material only certain horseflies and *Aedes* mosquitoes transmitted the virus 2 and 3 days after becoming infected.

Horseflies, deerflies, stable flies and mosquitoes punctured the egg membrane and engorged. It was hard to distinguish the punctures of black flies on the air sac membranes even under the microscope and there was no evidence that the horn flies attacked the membrane. The mouthparts of a stable fly, horsefly and mosquito in comparison with a 27 gauge hypodermic needle, all at a magnification of approximately 18×, are shown in figure 3. The mouthparts of the horsefly and deerfly which are similar produced two types of wounds on the inner shell membrane, one was a slash about one-fourth inch in length produced by the stylets and the other a white appearing area of scarification oval or circular in shape. Presumably the scarification was produced by the labella. The broad end of this organ with its numerous pseudotracheae would be an excellent means of mechanical transmission. The bundle of structures that comprise mosquito mouthparts are smaller than the 27 gauge needle, and of them only the delicate hair-like stylets to be seen to one side of the labium probed deep in search of blood. On occasions, the mosquito was seen to pump in clear allantoic fluid; then, with a change of position, tap a blood vessel. The blood was layered on the clear liquid in the distended abdomen of the insect.

Since infection occurs readily when virus is placed upon the chorioallantoic membrane, it was expected that if virus could pass through the shell membrane it would initiate infection. Contaminated house flies placed directly upon the opened egg did not induce any deaths. Virus was sprayed or dropped in 0.1 to 0.5 ml quantities upon the unbroken shell membrane in the air sac. Among 20 embryos exposed to more than $10^{6.6}$ LD_{50}'s of virus in this manner, only one death, which was nonspecific, occurred. On the other hand,

TABLE 3.—*Percent mortality of chicken embryos following exposure to VSV infected and noninfected diptera.*

Insect group	Insects exposed to virus		Insects not exposed to virus*	
	Number of embryos exposed	Percent mortality	Number of embryos exposed	Percent mortality
Horsefly	192	66	47†	11
Deerfly	76	57	25	8
Mosquito	75	53	17	0
Stable fly	74	51	42	7
Black fly	41	29		
Horn fly	21	0		

* Some insects were exposed to inactivated virus.
† This group includes 22 seven day embryos, others were 9 or 10 days.

the merest prick with a minuten nadenl dipped in virus was enough to initiate a fatal infection. One hundred and thirty-one control tests were made in which insects that had never been exposed to virus were placed upon embryonating eggs in the same manner as in the transmission trials (table 3). Only 8% of the embryos bitten by exposed insects died, as contrasted with 54% of the embryos which died within 24 hours after trauma by an exposed insect.

Deaths were caused, in some instances, by bacterial and mycotic contamination. One and 2% solutions of sodium propionate were used as a mold deterrent with moderate success. Embryos would tolerate solutions containing up to 5% by weight of the propionate when it was sprayed upon the shell membrane.

With the evidence that certain species of diptera could transmit VSV by producing an abrasion, the length of the survival of the virus in the vector remained to be studied. Mosquitoes were fed on blood-virus mixtures or upon virus-infected young chickens, and then isolated, fed only fruit juices or sugar solutions, until sacrificed by freezing at −20 C, triturated and inoculated into embryonating eggs. Virus-engorged mosquitoes when macerated with a needle and the needle employed to prick the membrane of embryonating eggs were shown to be highly infective. In a series of trials individual *Culex quinquefasciatus* mosquitoes were macerated. The tissues from each one with penicillin and streptomycin added were inoculated into 5 eggs by pricking the chorioallantoic membrane with the point of a 27 gauge needle that had been dipped into the mixture. Normal mosquitoes fed on uninfected chicken blood, sacrificed and injected in a similar manner were not lethal for the embryonating eggs. Positive results were obtained with infected mosquitoes up to 3 days and equivocal results for 5 to 7 days. Using nutrient broth containing 250 units of penicillin and 250 μg of streptomycin per 0.1 ml as the diluent a pool of 20 infected mosquitoes was found to contain the virus even at a titer of 10^{-3} two days after infection. The end point was not determined. A pool of *C. pipiens* was found to harbor the virus up to 7 days and possibly 14 days.

The possibility of transovarial transmission among stable flies and mosquitoes was investigated. Stable flies reared from the egg stage in the laboratory were fed on virus suspensions. Their eggs were kept separate and when they had developed into adults residual virus was determined by allowing them to bite susceptible embryos or by trituration of the insect and inoculation of the suspension into eggs. No evidence of the virus was found. Then *C. quinquefasciatus* females induced to engorge on infected chicken eggs were placed in a cage with males and two egg rafts were secured. Neither was fertile. A second trial produced viable ova. Trituration of eggs or of the larvae, pupae, and adults which developed failed to demonstrate the virus. The larvae of *C. quinquefasciatus* which were washed in distilled water and placed in tubes containing virus in dilutions from 10^{-1} to 10^{-4} grew like the controls. The virus

in dilutions from 1:10 to 1:28 in broth also had no effect. The virus infectivity of all the fluids in which larvae were placed was lost within 24 hours.

In the final experiments to determine whether diptera could infect cattle, 25 horseflies, 35 stable flies and 40 mosquitoes which had been exposed to VSV were allowed to bite 2 cows. The horseflies were collected in the Tomah, Wisconsin area, approximately 100 miles north of Madison. They included the following species: 2 *Tabanus lineola* Fabricus, 3 *T. lineola* var. *scutellaris* Walker, 1 *T. trispilus* Weidemann, 1 *T. reinwardtii* Wiedemann, 3 *T. quinquevittatus* Wiedemann, 1 *T. typhus* Whitney, 1 *T. trepidus* McDonnough, 3 *T. spp.*, 2 *Chrysops montana* Osten Sacken, 1 *C. frigida* Osten Sacken, 7 *C. spp.*

The tabanids were inoculated per os with virus 12 hours previous to the transmission tests. Thirty-five *S. calcitrans* from a laboratory reared strain, and probably half of the individuals of 20 *C. pipiens* and of the 20 *C. quinquefasciatus* were infected 24 hours before by feeding upon chickens.

Two Holstein cows from the University herd, in apparent good health and in lactation, were used as hosts. They were kept separate from other animals in a screened building during and after the trials. Serum samples which were taken from both cows prior to the test did not contain specific VSV antibody. Cow no. 28 was restrained by injection of chloral hydrate. Tabanids were placed on lips, feet and teats. Only one *T. trepidus* fly, held to the right front teat made a known bite. This fly bit twice in that area, causing the blood to flow copiously. It was then moved to the lips, but biting was not observed again. A similar procedure was followed with cow 199. Stable flies were applied to the lips on one side and mosquitoes to the lips, teats and feet on the other. Biting was not observed in any instance.

Cow 199, exposed to the mosquitoes and stable flies did not eat well for a week after the trial and lost some weight. Cow 28 exposed to the tabanids appeared normal. Lesions of stomatitis were not seen in either cow. Cow 199 showed no change in antibody titer but cow 28 developed neutralizing antibody to VSV. Ten days after exposure her serum neutralized $10^{2.5}$ LD_{50}s of the virus.

DISCUSSION

Laboratory demonstrations of arthropod transmission of disease must be interpreted cautiously. Although poliomyelitis can be transmitted by stable flies,[14] it does not appear to be pertinent to the natural history of the virus. On the other hand, early work on the transmission of the encephalitides in the laboratory[15] was confirmed later by field observations.[16]

Vectors may spread animal viruses in two ways. Biological transmission is characterized by a high degree of vector specificity, by failure to infect by interrupted feeding and by an incubation period during which the virus proliferates in the vector and before it reaches a portal of exit. Mechanical transmission is characterized by little vector specificity and by absence of incubation period.

Applying these criteria to the work with vesicular stomatitis, it is apparent that the transmission was mechanical. Several species of mosquitoes, deerflies and horseflies were able to transmit the disease to the embryonating eggs and to harbor the virus in an infective state.

14. Anderson, J. F. and Frost, W. H. 1912, Pub. Health Rep. **27**: 3–5.
15. Kelser, R. A. 1933, J. Am. Vet. M. A. **82**: 767.
16. Hammon, W. M., Reeves, W. C. and Galendo, P. 1945, Am. J. Hyg. **42**: 299–306.

No ovarian transmission or proliferation in the larval stages occurred. Inoculation by needle prick transferred the clinical disease in several laboratory and domestic animals. Interrupted feeding, both immediate and after several days, effectively transmitted the virus. No evidence of an incubation period or of an increase of virus in the insect was discovered. These results were not unexpected, since vesicular stomatitis virus resembles both fowl pox and myxomatosis, which are transmitted mechanically, in its predilection for the epidermis. In fowl pox[17] and myxomatosis[18] transmission takes place from lesions; intravenous feeding alone will not suffice. Since the viremia of vesicular stomatitis in cattle is of a low order,[6] it is probable that transmission occurs only when insects feed on lesions or contaminated sites. The attempt to transmit VSV to cattle by diptera was not very impressive as the clinical disease was not reproduced. It should be emphasized, however, that an egg-adapted strain of virus was employed. The attenuating effects of the passage of virus through other than the original hosts on transmission studies has been stressed by Kelser.[19] A monkey-adapted strain of equine encephalomyelitis could not be transmitted to guinea pigs by *Aedes aegypti* Linn. mosquitoes nor guinea pig-adapted strain to monkeys, although the strains were readily transmitted to the homologous hosts. It seems reasonable that the cattle-adapted strains isolated during an epizootic would be more readily transmitted by vectors than an egg-adapted strain.

17. Diegler, I. J., Muckenfuss, R. S. and Rivers, T. M. 1929, J. Exper. Med. **49**: 649.
18. Fenner, F., Day, M. F. and Woodroofe, G. M. 1952, Australian J. Exper. Biol. **30**: 139–152.
19. Kelser, R. A. 1951, Vet. Ext. Quart. Univ. Pa. **51**: 31–43.

There is not enough evidence to select the probable vector from the group of possible vectors. Any of the insects which transmitted the virus experimentally could traverse the distances between infected herds in northern Wisconsin and with reasonable rapidity. Jenkens and Hassett[20] refer to *Aedes* species in Alaska which infiltrated an area 16 miles square within 13 hours. Reeves and coworkers[21] found that the flight range of *Culex tarsalis* Coq. and *C. quinquefasciatus*, two species able to carry VSV in this study, to be as far as 2.5 miles. Western species of Simuliidae with flight ranges up to 7.4 miles in one day have been recorded.[22] MacCreary[23] records eastern species of tabanids as travelling 3 to 8 miles in one day.

Horseflies, deerflies, stable flies, and mosquitoes were collected in large numbers from cattle. Differences exist among horseflies in the amount of biting which they do and in their capacity to spread disease. Mohler and Thompson[25] found that *Tabanus atratus* Fabr. account for most of the spread of surra in Louisiana, although other species were present. The most important factor seemed to be the persistence and frequency of biting.

The geographical prevalence of biting diptera and their seasonal incidence are probably better clues to the species which take a significant part in the spread of VSV than the ability of a few individuals to transmit the virus in the

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21. Reeves, W. C., Brookman, B. and Hammon, W. M. 1948, Mosquito News, **8**: 61–69.
22. Dalmat, H. T. 1950, Ann. Ent. Soc. Am. **43**: 537–545.
23. MacCreary, D. 1940, Univ. Dela. Agric. Exper. Sta. Bull. 226.
24. Reeves, W. C. and Hammon, W. M. 1943, Am. J. Trop. Med. **24**: 131–134.
25. Mohler, J. R. and Thompson, W. 1911, Ann. Rep. Bur. Anim. Ind. U. S. Dept. Agric.

laboratory. The geographical limitation of VSV epizootics argues against the importance of an ubiquitous species like *S. calcitrans* as a vector or of some of the mosquitoes. The evidence now available as to the seasonal incidence of the tabanids which generally disappear before the epizootic gets underway suggests that they cannot be important. However, observations on the incidence of the horsefly has covered too few years and it is well to remember that VSV has occurred in Wisconsin at infrequent intervals. The possibility of late season abundance of these insects cannot yet be eliminated.

The abundance of certain mosquitoes and blackflies in late summer in regions in which outbreaks have occurred, their ability to transmit the virus, and to attack cattle must at present make them most suspected.

Definition of the transmission of VSV must await the isolation of the virus from diptera in the vicinity of an affected herd during an outbreak of the disease.

SUMMARY

A method of studying insect transmission of virus was developed, in which embryonating eggs were utilized as both the infective and susceptible hosts. This method was applied in some 700 trials to 28 species of biting diptera indigenous to Wisconsin.

The following species were able to transmit the virus of vesicular stomatitis under experimental conditions for 1 to 3 days: *Stomoxys calcitrans*, *Tabanus lasiophthalmus*, *T. trispilus*, *T. lineola* var *scutellaris*, *T. epistates*, *T. affinis*, *T. trepidus*, *Chrysops montana*, *C. striata*, *C. vittata*, *Aedes stricticus*, *A. stimulans*, *A. cinereus*, and *Culex tarsalis*.

Transfer of vesicular stomatitis virus by diptera appeared to be mechanical. Evidence of this lies in the number of species found to carry the virus, the failure to discover an extrinsic incubation period, and the rapid loss of ability to transmit the virus.

TYPING OF KLEBSIELLA BY INFRARED SPECTROPHOTOMETRY

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Serological procedures have been successfully used to segregate numerous capsule types within a single genus of microorganisms such as *Klebsiella*.[1] However, this procedure necessitates the preparation and stocking of the large number of type-specific serums required. In the case of the pneumococci[2] the possibility has been demonstrated of using infrared spectrums of the extracted capsular polysaccharides for the purpose of making a similar segregation. Of 57 polysaccharide types and subtypes examined spectrophotometrically, all but one pair of closely related subtypes were distinguishable. Polysaccharides of the same type obtained from different sources using different extraction procedures showed similar spectrums. These polysaccharides were highly purified, however, and the purification procedure still would be time-consuming and of questionable practicability.

Preliminary results indicated that the spectrums of whole cells were of little value in typing because of the variable ratio of capsular and somatic portions of the cell, and because of the variable occurrence of intracellular glycogen[3] and other substances, all of which contribute to the spectrum. In the case of organisms with large capsules it was thought that even crude extraction procedures would produce a capsular polysaccharide of sufficient purity to provide a method of type identification which would be practical at least for those laboratories having access to infrared spectrophotometric equipment. Crude products prepared in a few hours were used exclusively in this study. The data to be presented indicate that the spectrums of such crude capsular polysaccharides can be used for typing, and that spectral typing largely, but not completely, parallels serological typing.

EXPERIMENTAL

Capsular polysaccharides were prepared from cultures incubated approximately 18 hours at 37 C on the medium of Worfel and Ferguson.* Nutrient agar with 1% glucose did not provide comparable capsular development. The spectrums of preparations from cultures on both mediums were similar.

Diffuse surface growth was obtained by distribution of the inoculating suspension with a glass spreader. The capsule size was checked microscopically in an india-ink mount.[1] After the addition of a few milliliters of water, the growth was scraped from the surface by a rubber policeman with due care to avoid gouging the agar. Washing of the culture was omitted. Infrared spectrums are additive, so that any impurity from the medium will cause error if present in sufficiently large amounts. Impurities derived from the Worfel-Ferguson medium did not prove to be a serious obstacle. However, a synthetic medium of inorganic salts and glucose proved to be im-

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2. Levine, S., Stevenson, H. J. R. and Kabler, P. W. 1953, Qualitative studies of pneumococcal polysaccharides by infrared spectrophotometry. Arch. Biochem. Biophys. **45**: 65–73.
3. Levine, S., Stevenson, H. J. R., Tabor, E. C., Bordner, R. H. and Chambers, L. A. 1953, Glycogen of enteric bacteria. J. Bact. **66**: 664–670.

* Personal communication to P.R.E.: yeast extract 0.2%, sucrose 2.0%, sodium chloride 0.2%, magnesium sulphate 0.025%, potassium sulphate 0.1%, agar 2.0%, distilled water to volume.

practical because the polysaccharide product had to be dialyzed to remove contaminating salts.

The capsular polysaccharide was extracted with water. Extraction at 100 C for 15 to 30 minutes gave much better yields than extraction at room temperature or at 37 C. The growth from one or two Petri plate cultures yielded sufficient polysaccharide for test purposes. In addition, the extraction at 100 C partially or completely decapsulated the organisms so that subsequent centrifugation was facilitated. An angle centrifuge was used to remove the cells, frequently requiring 14,000 r.p.m. (25,000 g) in order to clarify the solution rapidly. A few drops of saturated sodium acetate were added and the polysaccharide was precipitated by four volumes of cold 95% ethanol. After standing a few minutes in the refrigerator the precipitate was removed by centrifugation, washed in cold 80% ethanol and recentrifuged.*

Films for spectral recording were prepared on silver chloride discs by dissolving the precipitate in a few drops of water, spreading and drying in air. The thickness of the film was adjusted by trial and error. Spectrums were obtained on a Perkin-Elmer model 21 double-beam spectrophotometer over the range 5 to 15 μ. The recording is automatic and accomplished in 15 to 20 minutes. When it was necessary to conserve material, a microcell adapter[4] was used to reduce sample requirements from 2.0 to about 0.4 mg. It was desirable to obtain spectrums of both thick and thin films in order to bring out maximum detail in the weakly and strongly absorbing regions of the spectrum. All of our products yielded smooth transparent films which scattered very little energy.

RESULTS

Interpretation of infrared absorption bands

The spectrums of all of the klebsiella polysaccharides contained bands at 6.2 and 7.1 μ, which are characteristic of the carboxylate ion (fig. 1b). It is probable that the carboxylate ion was present in the polysaccharides in the form of salts of uronic acids. Unlike the spectrums of pneumococcal polysaccharides,[2] there were no instances of polysaccharides which showed detectable amide bands (as found in acetylated amino sugars), or both amide and carboxylate bands, or neither set of bands. Thus it was impossible to divide the spectrums into four primary groups to facilitate identification.

The presence of sharp carboxylate ion bands at 6.2 and 7.1 μ was used as a criterion of a good preparation. If centrifugal removal of the extracted cells was incomplete, the spectrum showed evidence of amide bands at 6.05 and 6.45 μ, the former fusing with and broadening the 6.2 μ carboxylate band, the latter appearing as an inflection. These amide bands (unlike the amide bands seen in pneumococcal polysaccharides) were attributable to bacterial protein as seen in the spectrum of intact cells (fig. 1a), and are eliminated by proper centrifugation.

When the capsule as seen in india-ink mounts was small, the yield of the isolation procedure was much less than usual. The spectrum of such a product is presented in figure 1c. It may represent impurities extracted from the medium or the somatic part of the cell. Similar products were obtained from poorly encapsulated klebsiellas of several types. It is possible that all our preparations were similarly contaminated, but in favorable well-encapsulated cultures the yield of type-specific polysaccharide greatly overshadowed the nonspecific impurities. Consequently, the rapid polysaccharide isolation outlined above can be applied only to well-encapsulated cultures.

Many of the polysaccharide spectrums showed a band at 5.77 μ associated with bands at 7.25 and 8.0–8.2 μ, all of which are probably due to the acetyl groups bound to the sugar molecules in ester linkage. The origin of these bands and the effect of pH on the carboxylate ion bands have been dis-

* Preparations of type 1 and type 2 capsular polysaccharides reacted with specific serums in precipitin tests up to a dilution of 1:200,000.

4. Stevenson, H. J. R. and Levine, S. 1953, Holder for infrared spectrophotometry of small samples on silver chloride. Rev. Sc. Instruments **24**: 229–230.

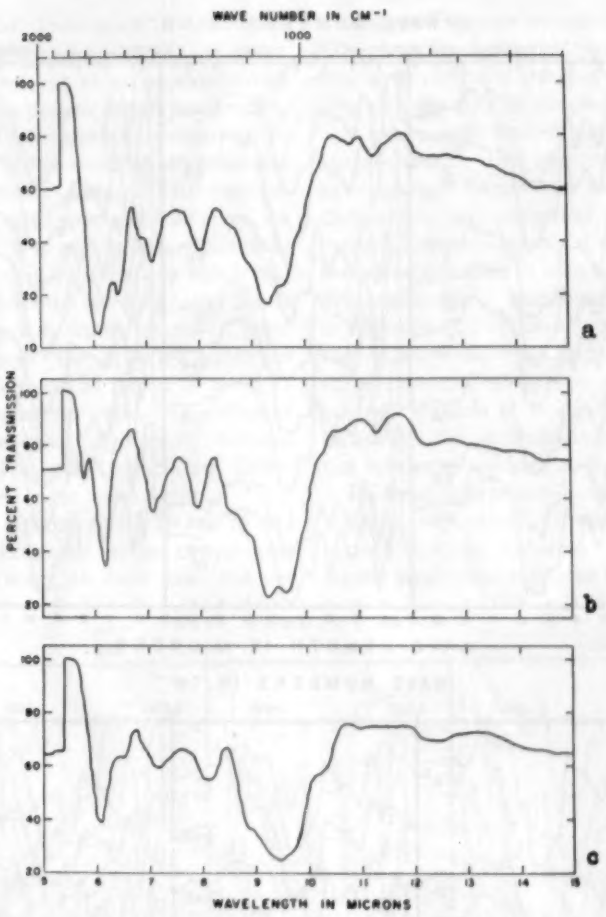


FIG. 1.—a.—Infrared spectrum of intact cells of klebsiella type 1. The contribution of the capsular polysaccharide can be recognized, but the carboxylate ion bands are overshadowed by the amide (protein) bands at 6.05 and 6.45 μ.

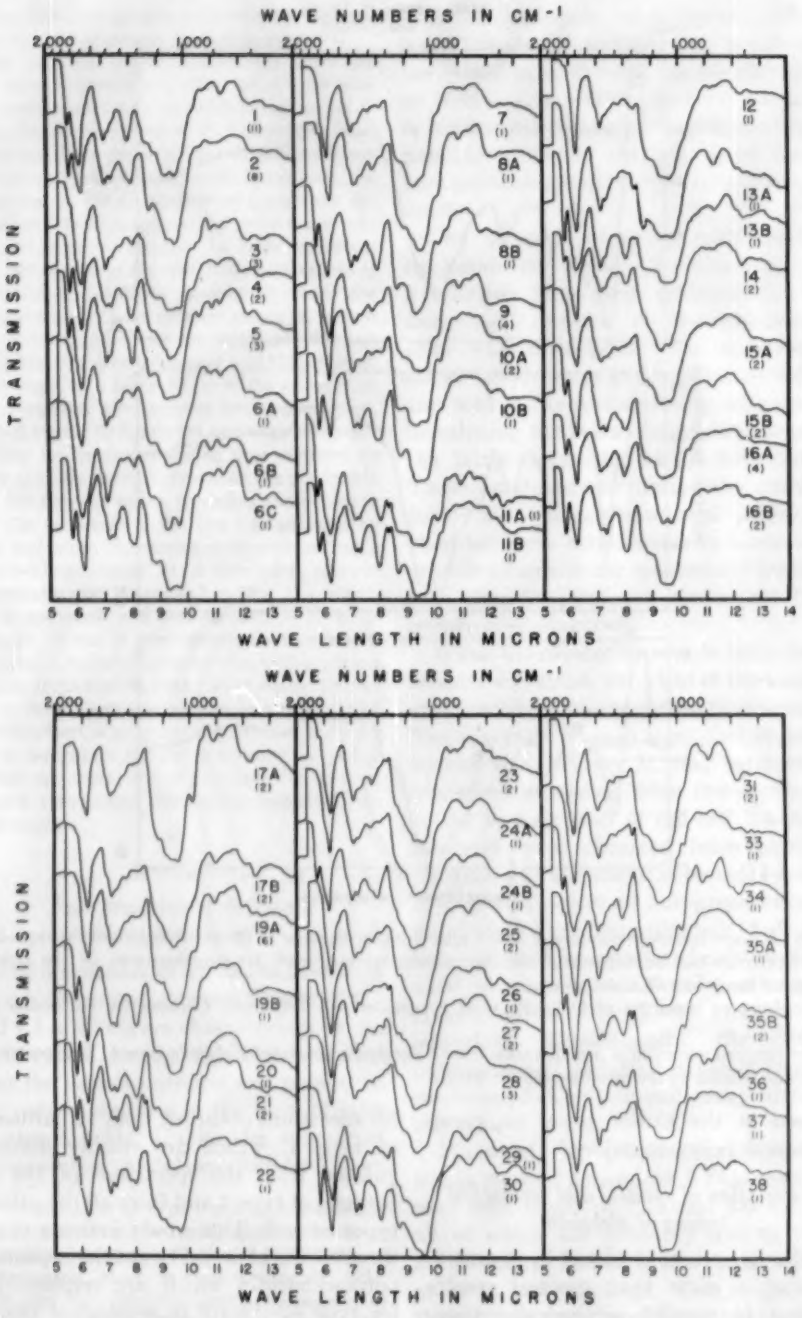
b.—Infrared spectrum of klebsiella type 1 capsular polysaccharide. Carboxylate ion bands at 6.2 and 7.1 μ.

c.—Infrared spectrum of a preparation from a poorly encapsulated klebsiella type 1. The spectrum shows no relation to the capsular polysaccharide.

cussed in the earlier paper on pneumococcal polysaccharides.[2]

Correlation of spectral and serological typing of klebsiella

The spectrums depicted in figures 2, 3, and 4 show that spectral results tended to parallel serological typing. This is best indicated by the very similar spectrums obtained from 11 cultures of type 1, which are readily distinguished from the spectrums of the 8 cultures of type 2 and from all the other types as well. This would indicate that those chemical properties of the capsular polysaccharides which are responsible for type specificity in serological reactions are reflected in the infrared spec-


WAVE NUMBERS IN CM⁻¹
2,000
1,000
TRANSMISSION
1 (1)
2 (8)
3 (3)
4 (2)
5 (3)
6A (1)
6B (1)
6C (1)
7 (1)
8A (1)
8B (1)
9 (4)
10A (2)
10B (1)
11A (1)
11B (1)
12 (1)
13A (1)
13B (1)
14 (2)
15A (2)
15B (2)
16A (4)
16B (2)
5 6 7 8 9 10 11 12 13 14
WAVE LENGTH IN MICRONS
WAVE NUMBERS IN CM⁻¹
TRANSMISSION
17A (2)
17B (2)
19A (6)
19B (1)
20 (1)
21 (2)
22 (1)
23 (2)
24A (1)
24B (1)
25 (2)
26 (1)
27 (2)
28 (3)
29 (1)
30 (1)
31 (2)
33 (1)
34 (1)
35A (1)
35B (2)
36 (1)
37 (1)
38 (1)
WAVE LENGTH IN MICRONS

trums. Slight differences in the spectrums of polysaccharides of the same type do occur but must be overlooked in favor of the preponderant similarities.

In several cases the spectrums of polysaccharides of the same serological type were sufficiently different to warrant separation into spectral subtypes. In these cases the spectrums apparently revealed chemical differences which had not been detected serologically (quellung and agglutination tests). In most of these cases the spectral subtypes showed differences in depth of the 5.77 μ band, probably indicating different degrees of acetylation. Usually, but not always, there were associated differences in the longer wave lengths.

Cultures of types 6, 10, 15, 19, 35 and 52 were investigated further by quellung absorption tests. In each case, the use of absorbed serums revealed serological differences among the cultures of a given type and confirmed the spectral division into subtypes, as illustrated in table 1.

It is apparent that in the wavelength range utilized, the spectrums do not have enough bands and inflections to distinguish an unlimited number of types and subtypes. Consequently, there are instances in which the spectral differentiation of two types is difficult or impossible. Frequently the types involved also show serological relationships.[1] Spectral intertype relationships are not capable of being translated in numerical and quantitative terms as are the results of serological tests.

Infrared spectrums have shed some light on serologically intermediate cultures, that is, cultures which show strong serological relations to more than

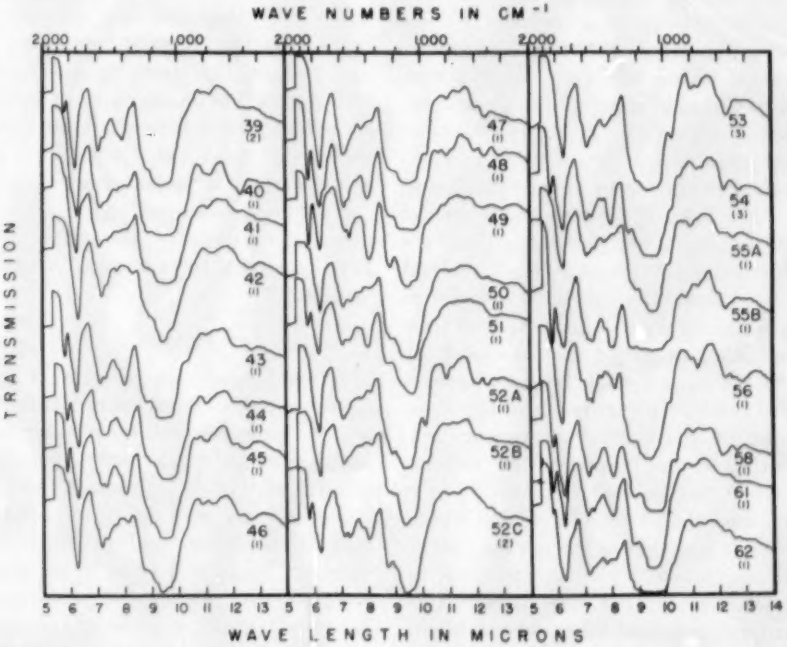


FIG. 2, 3 and 4.—Infrared spectrums of crude capsular polysaccharides prepared from well-encapsulated klebsiellas. The number indicates the serological type, the letter indicates the spectral subtype, and the number in parentheses indicates the number of strains from which such preparations were obtained.

TABLE 1.—*Quellung absorption tests with type 15 serum (Mich 61).*

Spectral subtype	Cultures	Unabsorbed	Absorbed by 4158–51	Absorbed by 1218–52	Absorbed by 3486–50
15A	4158–51	+++	–	–	–
15A	1218–52	+++	–	–	–
15B	3486–50	+++	+	+	–
15B	Mich 61	+++	+	+	–

one of the established types. Two cultures related strongly to both types 8 and 25 were found to yield polysaccharide spectrums indistinguishable from that of type 25 but readily differentiated from that of type 8. Serological tests also indicated the closer kinship to type 25 since the cultures reacted with type 25 serum and not with type 8 serum after the serums had been cross-absorbed.

DISCUSSION

The results presented indicate that capsular typing of klebsiellas by infrared spectrophotometry is possible. Spectral typing to a large extent parallels serological typing, although in some cases it appears to make finer distinctions than simple quellung and agglutination tests (spectral subtypes) and in other cases it fails to make certain distinctions. When typing is done for epidemiological reasons either method may be suitable since both can distinguish one strain of a given species from most other strains of that species. If an outbreak of a disease is to be investigated, simple comparison of the spectrums of polysaccharide preparations from different cultures will indicate whether the isolated organisms are of the same type. This information will be clinically useful even though the numerical designation of the type remains unknown. The probable serological type can be determined by comparison with the spectrums of known types which we have presented. The infrared method appears to be sufficiently rapid, requiring an overnight incubation and a few hours of subsequent isolation procedures after the original colony is picked from the isolation plate. It has the advantage of not requiring large numbers of specific serums. It is potentially a method of wide applicability—certainly to all the encapsulated bacteria—and preliminary results indicate the possibility of application to the somatic polysaccharides of noncapsulated organisms. The usefulness of the method would probably be increased by extending the spectral range into the far infrared (beyond 15 μ) and by reducing the sample size requirements with microscope attachments.

Infrared typing has certain limitations. We have imposed the limitation of large capsule size because of our insistence on a rapid and simple isolation procedure. A limitation may arise in some groups of organisms if the polysaccharide films are not transparent, but scatter light strongly, requiring the use of mineral oil mulls or other special techniques. The availability of infrared spectrophotometers is the most serious limitation at present. However, it may prove simpler to make arrangements to use an instrument belonging to an industrial or university laboratory, rather than prepare a large number of specific serums.

SUMMARY

Infrared spectrophotometry of crude capsular polysaccharides has been presented as a method for typing klebsiellas. Spectral typing largely paralleled serological typing, although in some cases it was necessary to establish spectral subtypes, and in other cases different serological types could not be resolved. The existence of subtypes has been confirmed by quellung absorption tests. The possibility of application in epidemiological work and the limitations of the method have been discussed.

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